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PRINCIPAL INVESTIGATOR: Andrea M. Allan, Ph.D.

CONTRACTING ORGANIZATION: The University of New Mexico
Albuquerque, New Mexico 87131-5041

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Andrea M. Allan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of New Mexico
Albuquerque, New Mexico 87131-5041

E-Mail: vcjordan@nwu.edu

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13. ABSTRACT (Maximum 200 Words)
Alcohol use has been identified as an important factor in aggressive, or violent, behavior in humans. Alcohol not only increases the incidence but also the severity of violent attacks. Several clinical studies have reported the observation that highly aggressive individuals display a serotonin-deficient trait. A number of studies indicate that the $5HT_3$ receptor system mediates alcohol consumption and the subjective effects of alcohol. The $5HT_3$ receptor is unique in the serotonin receptor family in that it is a cation channel and modulates the release of a number of other neurotransmitters, including GABA and dopamine. Thus, the 5-HT_3 receptor is likely to play a critical role influencing alcohol consumption, which appears to involve dopamine and influencing both natural and alcohol-heightened aggression through the $GABA_A$ receptor system. We have developed a 5-HT_3 receptor over-expressing mouse to study the role of this receptor in alcohol drinking and aggression. We hypothesize that $5HT_3$ receptor over-expression decreases alcohol preference and aggressive activity through a $5HT_3$ receptor sensitive mechanism increasing the release of GABA. We will evaluate the transgene on 3 different inbred strains (C57, DBA, 129) who differ in alcohol preference in a two-bottle choice design and aggression using intruder-aggression test. We will test the ability of $5HT_3$ receptor antagonists to block and GABA receptor agonist to mimic the phenotypic effects of over-expression. These mice should prove useful in testing hypothesis regarding the role 5-HT_3 receptors play in alcohol abuse and alcohol-heightened aggression.

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Introduction (Unmodified from original)

Alcohol use has been identified as an important factor in aggressive, or violent, behavior in humans. In its 10th Special Report to the U.S. Congress on Alcohol and Health (June 2000), the Public Health Service stated that one in four victims of violent crime described their attacker as having consumed alcohol prior to committing the crime. When the victim was a current or former intimate partner, the incidence increased to two out of three offenders having been drinking prior to the attack. Alcohol not only increases the incidence but also the severity of the attack. While these statistics may be correlational, a causal relationship between alcohol use and physical violence needs to be explored.

Experimentally, aggression in mice can be brought about by a isolating male for some time, then, subsequently, to introduce a group-housed male to the isolated male's home cage (*isolation-induced aggression or intruder-aggression*), with heightened attacks seen when the resident mouse is an actively breeding male (Malick, 1979; Miczek, 1999). Many environmental and experiential variables factor into aggressive behavior, but the administration of alcohol is an important societal variable in aggressive behavior (Miczek et al., 1998). Several lines of research on the neurochemical basis for aggression have implicated the serotonin system (Korte et al., 1996; Fish et al., 1999; DeAlmedia et al., in press). Alterations in the serotonin system, using knock out technology, have produced lines of mice that display altered aggressive behavior (Saudou et al., 1994; Cases et al., 1995). Pharmacologic studies have implicated the involvement of 5-HT_{1a} (deBoer et al., 2000), 5-HT_{1b} (Fish et al., 1999) and 5-HT_3 (Rudissaar et al., 1999) receptors. Several clinical studies have reported the observation that highly aggressive individuals display a serotonin-deficient trait, as measured by either low levels of cerebral spinal fluid (CSF) 5-hydoxyindole acetic acid (5-HIAA) or a flattened prolactin response to serotonin activation (Brown et al., 1982; Linnoila et al., 1983; Coccaro et al., 1990, Mann, 1999). While these data provide support for a role for serotonin in aggression, there is strong support for gamma amino butyric acid (GABA) as well (Soderpan and Svensson, 1999; Glaysheva et al., 1998; Guillot et al., 1998; Navarro and Pedraza, 1996; Miczek et al., 1995; 1994,1993; Weerts et al., 1993).

Besides the suggestion that serotonin plays a role in aggression, several lines of research have suggested a role for serotonin in the regulation of alcohol consumption. A number of studies indicate that the $5HT_3$ receptor system mediates alcohol consumption and the subjective effects of alcohol. $5HT_3$ receptor antagonists decrease alcohol intake in laboratory animals (Jankowska et al., 1995, Jankowska et al. 1994, Tomkins et al. 1995, Knapp and Pohorecky 1992, Hodge et al. 1993) and humans (Johnson et al., 1993; Sellers et al., 1994). In addition, $5HT_3$ receptor antagonists increase the subjective feeling of alcohol intoxication in humans (Swift et al., 1996), suggesting that $5HT_3$ receptors are involved in alcohol sensitivity. Lovinger and his colleagues have shown that alcohol directly potentiates the $5HT_3$ receptor (Lovinger, 1991; Lovinger and White, 1991; Lovinger and Zhou, 1994). The $5HT_3$ receptor is unique in the serotonin receptor family in that it is a cation channel and modulates the release of a number of other neurotransmitters, including GABA and dopamine. Thus, the 5-HT_3 receptor is likely to play a critical role influencing alcohol consumption, which appears to involve dopamine and influencing both natural and alcohol-heightened aggression through the $GABA_A$ receptor system.

We hypothesized that over-expression of 5-HT_3 receptors decreases alcohol consumption because the presence of an increased number of 5-HT_3 receptors increased the potentiation of dopamine release at lower alcohol concentrations. Thus, the animal requires less alcohol to obtain the same behavioral effect. Alternatively, it is possible that the over-expression of 5-HT_3 receptors increases the release of gamma aminobutyric acid (GABA), an inhibitory neurotransmitter. GABA plays an important role in the inhibitory circuit from the prefrontal cortex to the amygdala and ventral striatal areas. This pathway is thought to play a role in moderating impulsive and compulsive behaviors. Thus, the lower level of alcohol consumption seen in the 5-HT_3 receptor over-expressing mice may be the result of increased inhibitory control over alcohol consumption. That is, these transgenic mice know "when to say when". To add support for this hypothesis, we found that the 5-HT_3 receptor over-expressing mice fail to behave aggressively in an intruder aggression test. The wild type mice clearly displayed high levels of aggression by attacking the intruder within seconds of introduction and continuing the attacks until the test was halted. This lower level of aggression in the 5-HT_3 receptor over-expressing mice may be due to an increase in GABA activity. The proposed studies will explore the neurochemical relationship between the lower alcohol consumption and lower levels of aggression seen in 5-HT_3 receptor over-expressing transgenic mice. The first goal will examine the impact of $5HT_3$ receptor over-expression on alcohol preference using a two-bottle free choice test. The second goal will examine the impact of $5HT_3$ receptor over-expression on natural aggressive behavior. Lastly, the impact of $5HT_3$ receptor over-expression on alcohol-heightened aggressive behavior will be measured.

Body

Progress made towards our goals are as follows: Breeding onto the three different backgrounds has been completed. Generations N1, N3 and N5 are the tested generations planned. We have completed generation N1 and N3 and a portion of the testing for the N5 generation. We have additional data on impulse control in a novel object maze task as well as elevated plus maze behavior.

Lower Alcohol Drinking Phenotype Conserved On Various Genotypic Backgrounds in the first generation of cross breeding

Reduced alcohol preference as a result of $5HT_3$ receptor over-expression was conserved even when expressed on a genetic background known for high alcohol preference, C57BL/6J (Figure 1). Using a two-bottle free choice test, C57BL/6J mice drank an average of 14 g/kg/day, while the Line 13 x C57BL cross transgenic mice drank an average of 8 g/kg/day, while the 129 mice drank an average of 8 g/kg/day the line13X129 drank 3.75 g/kg/day. The transgene did not influence the DBA drinking which remained below 1g/kg/day.. **Thus, the presence of the transgene was able to influence alcohol preference on a different genetic background even though these mice are heterozygous for the transgene.**

As depicted below in Figures 1 and 2 below, the influence on the transgene to reduce alcohol drinking in high alcohol consuming strains is maintained on the first generation background. Data from the N3 and N5 generations suggests that this influence is still present, on the C57BL/6 background. Additionally, there continues to be a lack of effect in the DBA mice. This is expected since the DBA mice do not consume ethanol and the transgene does not alter this phenotype. We did not move the transgene past the N1 generation on the 129 mice since the C57 background information proved to be reliable.

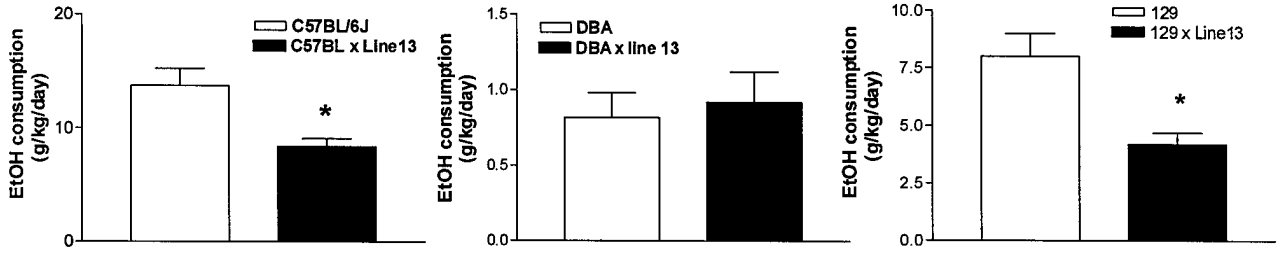


Figure 1 *Previously reported data* *Ethanol consumption averaged over 10 days in a two bottle free choice test in C57BL/6 xLine13 cross compared to C57BL/6 mice (left panel), DBA/2J vs. DBA/2J x line 13 cross mice (center panel) and 129/J xLine13 cross compared to 129/J mice (right panel). Data are mean ± SEM, n=10. Asterisk indicates significance at p<0.05.*

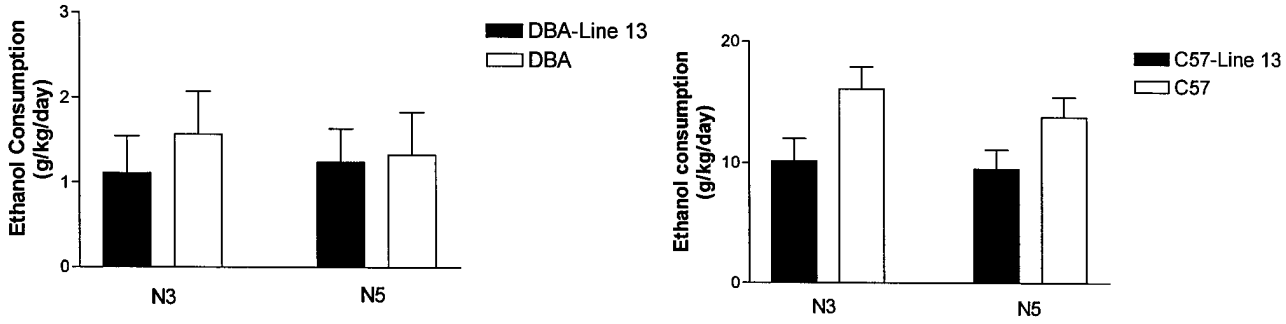


Figure 2 *New data* The effect of the 5HT3 receptor over-expression on the DBA(left panel) and C57 (right panel) inbred background strain from the 3 and 5th generations of the backcross. Comparison of the daily amount of ethanol consumed during a two bottle choice of drinking of 10% w/v ethanol in water vs water alone.

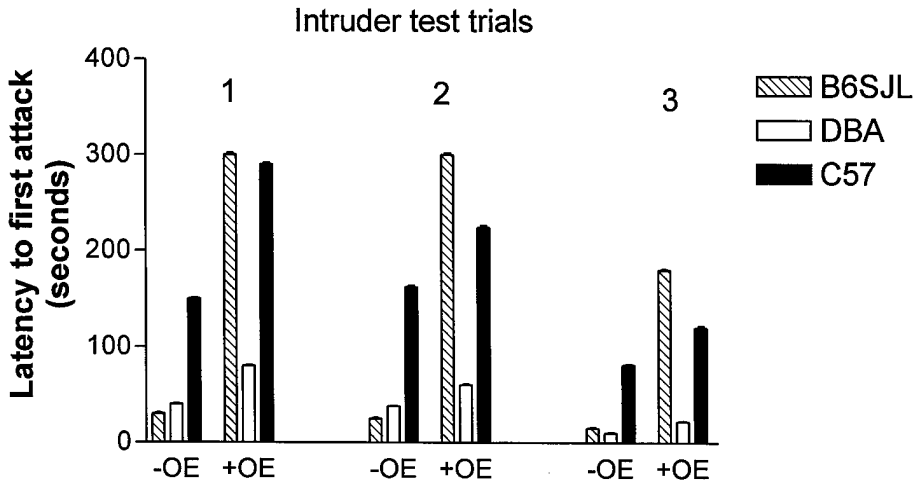


Figure 3 *New data* Intruder aggression *test. Latency to attack in seconds. The graph lists three trials over a consecutive 3 day period. Three different strain backgrounds are presented with (+OE) and without (-OE) the 5HT3 receptor transgene present. Data are mean +/-SEM, n=7 mice. Male group housed A/J mice served as intruders. Residents housed singly 2 week prior. Testing was performed in the resident's home cage.* ***Over-expressing mice (+OE) who failed to attack during the 5 minute test period ,were given a score of 300 seconds.***

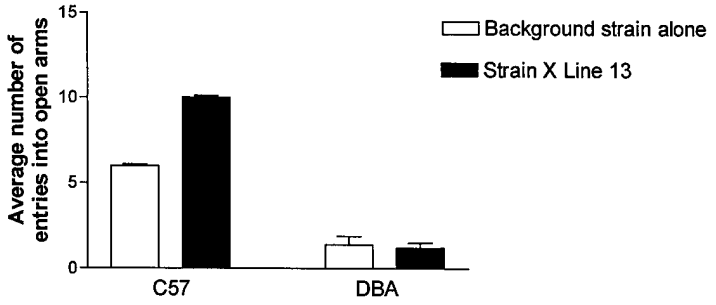
5-HT$_3$ Receptor Over-Expression Significantly Lowers Intruder Aggression

Using a standard intruder test, we evaluated the level of aggressive behavior in the transgenic 5-HT$_3$ receptor over-expressing mice. Resident mice were isolated for one week prior to the testing. A/J male group housed mice were used as the intruders. The intruder mouse was placed bite. If no biting took place the test was terminated after 5 minutes. Thus, the longest the test was run would have been 10 minutes. In this way we avoid any bias against aggressive behavior that may begin later in the testing period but yet can still be very intense in nature. Further measurements of latency alone may distort the measures of actual fighting once it is initiated.

Mice who over-express the 5HT3 receptor (+OE) generally failed to engage in biting or other forms of attack (Figure 3). These were single tests and these were not breeding males. Repeated testing and breeding male tests reveal a different picture. In repeated engagements, transgenic mice did attack the intruder with a greater frequency and more quickly than on the first exposures. We have measured alcohol-heightened aggression in preliminary tests. While the transgenics do show an increase in aggressive behavior with alcohol, aggression is still significantly lower than in the control mice, with an average of 3.5 attack bits for transgenics and 11 attack bites for controls after 1 g/kg alcohol injected i.p. 10 minutes prior to testing. Data on aggression following free access alcohol are preliminary and additional animals need to be studied. Further we are measuring the effect of repeated encounters in the resident's cage.

5-HT$_3$ Receptor Over-Expression Significantly Lowers anxiety

We began to notice that the Line 13 5HT3 receptor over-expressing mice display lower levels of anxious behavior compared to the wild type. While we did not follow this out across all the generations the most important generation to test would be the N5. If the background strain were to dilute out the anxiogenic effect of the transgene then the greatest dilution would be seen in generation 5. Using the elevated plus maze we measured the number and duration of entry into the open arms of the maze. Entry into these arms indicates lower anxiety states since these arms are designed to be clear and have no walls. We found no difference in the number of entries or in the time spent in the closed arms of the maze, indicating that the difference seen in the open arms was not the result of differences in general locomotion among the strains. DBA mice, in general, are more fearful and less mobile in this maze than are the C57 mice. The presence of the transgene on the C57 background (C57 x Line13) increased open arm entry and time spent in the open arms significantly over the control mice (C57). An additional test of anxiety was selected and run on a separate group of 5HT3 receptor over-expressing C57 and DBA mice from the N5 backcross generation. The novel object test takes place in an open field arena. The mouse is allowed to explore the arena for 5 minutes then a novel object is placed in the center of the arena. Mice generally avoid both the center of an open field and novel objects. Entry into the center area (a 3.5 inch diameter circle) in near proximity to the novel object (0.5 inch cube) indicates lower anxiety and less behavioral inhibition. Similar to the elevated plus maze, the C57 mice display much less anxiety compared to the DBA mice. However, there was a similar level of curiosity and willingness to approach the novel object in the two background strains. Over-expression of the 5HT3 receptor increased the time both strains spent near the object but increased number of center entries only on the C57 background. Thus, using a different measure we were able to identify an effect of the transgene on the DBA strain as well as validate the findings of the elevated plus maze for the C57 strain.



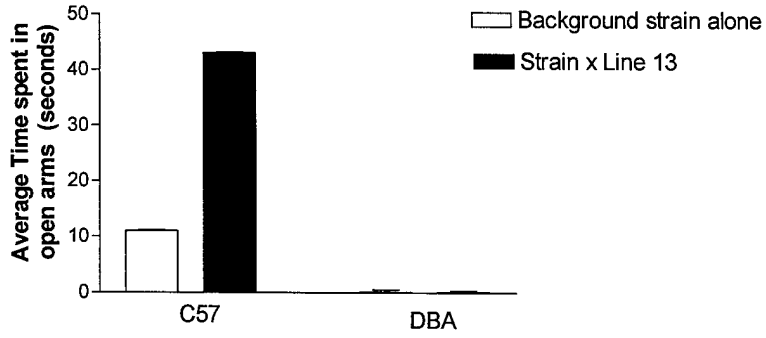
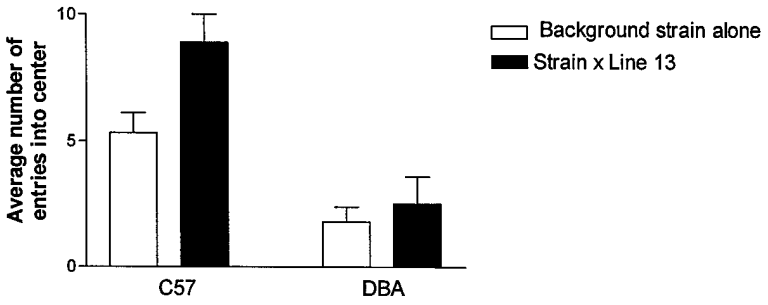


Figure 4: Entries into open arms and time spent in open arms of an elevated plus maze in control strains (C57 or DBA, open bars) compared to the 5HT3 receptor over-expressing gene on the two inbred strain backgrounds (C57 and DBA, closed bars). A significant decrease in anxious behavior was seen in the C57Bl mice with the 5HT3 receptor over-expression. There was no effect on maze performance when the gene was expressed on the DBA background.



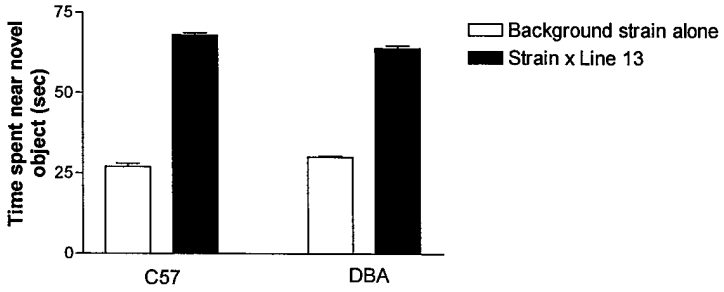


Figure 5: Entries into center and time near the novel object in control strains (C57 or DBA, open bars) compared to the 5HT3 receptor over-expressing gene on the two inbred strain backgrounds (C57 and DBA, closed bars). A significant decrease in anxious behavior was seen in the C57Bl mice with the 5HT3 receptor over-expression indicated by the increase center entries. Interestingly the 5HT3 receptor over-expression did significantly increase time spent with novel object for the DBA mice. Indicating the 5HT3 receptor may play a role in inquisitive behavior .

5-HT$_3$ Receptor Over-Expression Significantly Improves Learning

As an additional measure of impulse control we have been examining learning using a fear conditioned freezing measure with these lines. Fear conditioning is a classical Pavlovian learning measure where the mouse learns to anticipate a foot shock through the association of a preceding tone and the learning context itself. Learning is indicated by freezing. Over-expression of the 5HT3 receptor significantly improves learning in this system on the B6SJL mouse background (see figure 6). These data suggest that the over-expression of the 5HT3 receptor permits better stimuli association and information processing but is strain dependent. Additionally, these results indicate that over-expression of the 5-HT3 receptor in mouse forebrain results in enhanced hippocampal-dependent learning and attention.

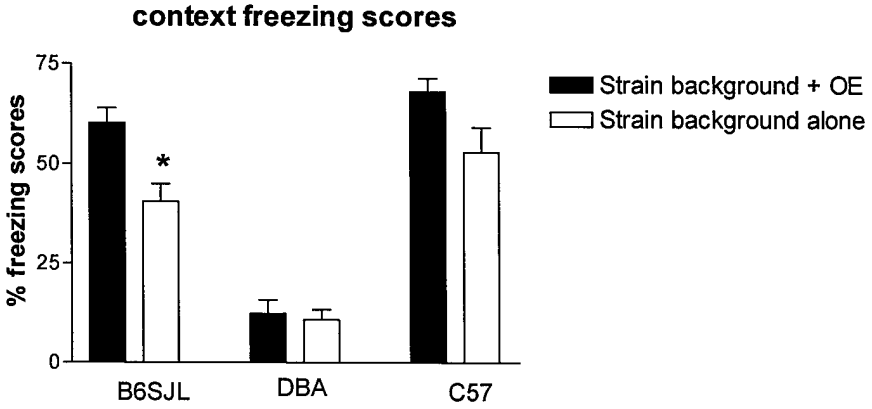


Figure 6: *New data* Percent time spent freezing during the context test following fear conditioning. Over-expression of the 5HT3 receptor improved learned freezing behavior in the B6SJL line but did not enhance learned freezing on the other strain backgrounds.

Key Research Accomplishments

- Breed the transgene on to the background strains out for 5 generations.
- Test the two bottle choice drinking on the wild type and transgenic N3 and N5 generations of the two strains.
- Test initial and repeated aggressive behavior on the wild type and transgenic N1-N3 strains.
- Identified an effect of the 5HT3 receptor on anxiety related behavior.
- Characterize unique learning and stress responding in the mice which impacts consumption and aggression.
- Began alcohol induced aggressive behavior testing on the N1 strains.

Reportable Outcomes

I.Y. Choi, **A.M. Allan**, and L.A. Cunningham Moderate fetal alcohol exposure (fae) mitigates the effects of enriched environment on adult hippocampal neurogenesis in mice., Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131 Society for Neurosciences 2003

A, M Allan, R. D. Paz and K.K. Caldwell Mouse fetal alcohol exposure impairs hippocampal-dependent learning. University of New Mexico, Albuquerque, NM, 87131-5223. Research Society on Alcoholism 2003

Allan, AM, Chynoweth, J and Caldwell,K K "A Moderate exposure fetal alcohol mouse model using saccharin fading technique." Research Society on Alcoholism 26:531, 2002

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Harrell, A.V. and **Allan, A.M**. Improvements in Hippocampal-Dependent Learning and Decremental Attention in 5-HT3 Receptor Over-Expressing Mice Learning and Memory, in press, Oct/Nov issue (2003).

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Conclusions

At least a generation 1 of the cross breeding the original impact of the transgene appears stable. However, as the background becomes more inbred the influence seems to be diluted. Further, the impact of the transgene on reducing aggressive behavior is upon initial exposure to the intruder but is overwhelmed but repeated presentations of the threat. This suggests a learned component or plasticity involved in aggressive responding that has not been noted in the literature at this time. The transgene appears to lower anxiety related behavior (novelty exploration is elevated and time spent in open arms of the elevated plus maze is increased) and this effect is somewhat strain background dependent. Future studies will measure the effect of the transgene on the perseveration of responding in an active avoidance paradigm.

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Improvements in Hippocampal-Dependent Learning and Decremental Attention in 5-HT_3 Receptor Over-Expressing Mice

Amber Viola Harrell and Andrea M Allan
University of New Mexico School of Medicine
Health Sciences Center, Department of Neurosciences
Albuquerque, New Mexico 87131 USA

5-HT_3-OE mice: improved learning, attention

5-HT_3 receptor, fear conditioning, latent inhibition, sensorimotor gating, novelty, anxiety

Amber Viola Harrell
University of New Mexico School of Medicine
Health Sciences Center, Department of Neurosciences
Basic Medical Sciences Building, Rm 145
Albuquerque NM 87131-0001
Phone: (505) 272-4447
Fax: (505) 272-8082
E-mail: ametz@salud.unm.edu

This manuscript has been seen and approved by all listed authors.

Suggested names of qualified referees:
Carol A. Barnes, Michael Davis, Joseph Le Doux and Nick J Mackintosh

Abstract

The 5-HT_3 receptor for serotonin is expressed within limbic structures and is known to modulate neurotransmitter release suggesting that this receptor may influence learning and memory. Perturbations in serotonergic neurotransmission lead to changes in the ability to attend, learn and remember. To examine the role of 5- HT_3 receptors in learning, memory and attention, 5-HT_3 receptor over-expressing (5-HT_3-OE) transgenic mice and their wild type litter-mates (WT) were tested in Pavlovian contextual and cued fear conditioning, fear extinction, and latent inhibition (LI) paradigms. Prepulse inhibition (PPI) was also assessed to reveal changes in sensorimotor gating. Additionally, anxious behaviors, shock sensitivity and reactions to novel stimuli were evaluated. 5-HT_3-OE mice displayed enhanced contextual conditioning, while cued conditioning remained the same as that of WT mice. 5-HT_3-OE mice did not differ from WT in extinction rates to either the context or cue. LI was enhanced for 5-HT_3-OE mice compared to WT. PPI remained unchanged. No differences in sensitivity to footshock or startle were found. However, 5-HT_3-OE mice demonstrated heightened exploratory behavior in response to novel environmental stimuli and decreased anxiety as measured in the elevated plus-maze. Results indicate that over-expression of the 5-HT_3 receptor in mouse forebrain results in enhanced hippocampal-dependent learning and attention. Decreased anxiety and enhanced exploratory behavior in response to novelty may contribute to the observed improvements in learning, memory and attention due to 5-HT_3 receptor over-expression.

Introduction

Of the seven classes of serotonin receptors, the 5-HT_3 receptor is the only ionotropic receptor found in vertebrates (Derkach et al. 1989; Maricq et al. 1991; Boess et al. 1995). Only two subunits, 3A and 3B, are currently known (Hope et al. 1993; Werner et al. 1994; Davies et al. 1999; Dubin 1999; Miquel et al. 2002). Of these, only the 3A subunit is expressed within the rat brain where it can form a homomeric receptor (Morales and Wang 2002 but see Monk et al 2001) and may co-assemble with nicotinic α4 receptor subunits (VanHooft et al. 1998; Kriegler et al. 1999; Nayak et al. 2000 but see Harkness and Millar 2001). Sharing up to 30% sequence homology with the nicotinic acetylcholine receptor, the 5-HT_3 receptor belongs to the superfamily of ligand-gated ion channels (Gurley and Lanthorn 1998; Ortells and Lunt 1995). Like the nicotinic receptor, the 5-HT_3 receptor is cation permeable. Ligand binding results in channel opening and allows for the flux of Na^+ and Ca^{2+} (Kriegler et al. 1999; McMahon and Kauer 1997; Nayak et al. 1999).

5-HT_3 receptor mRNA and binding sites are found in abundance in the rodent forebrain, namely the entorhinal cortex, piriform cortex, amygdala, and hippocampus. (Kilpatrick et al 1987; Barnes et al. 1990a; Gehlert et al. 1991; Cicinsain and Jenner 1992; Ohno and Watanabe 1997; Tecott et al. 1993; Doucet et al. 2000; Bloom and Morales 1998; Nayak et al. 1999). The 5-HT_3 receptor can be localized presynaptically as an autoreceptor, but more commonly in the hippocampus, amygdala and cortex, it is located postsynaptically on presynaptic terminals (MacDermott et al. 1999; Barnes and Sharp 1999; Khakh and Henderson 2000 but see VanHooft and Vijverberg 2000). This localization allows for the modulation of release of neurotransmitters such as dopamine,

norepinephrine, acetylcholine and GABA (Campbell and McBride 1995; Campbell et al. 1996; Allan et al 2001; Matsumoto et al. 1995; Consolo et al. 1994; Giovannini et al. 1998; McMahon and Kauer 1997; Ferezou et al. 2002). The 5-HT_3 receptor is, thus, in a position to exert modulatory control over neurotransmission in areas involved with learning and memory, in particular the hippocampus and amygdala (Schafe et al. 2001; Maren 2001).

Perturbations in serotonergic neurotransmission lead to changes in the ability to attend, learn and remember. For example, depletion of dietary tryptophan, the amino acid required for serotonin synthesis, causes impairment in the consolidation of memory during working memory tasks (Rubinsztein et al. 2001). Furthermore, MAO-A deficient mice have elevated serotonin levels and enhanced classical fear conditioning, indicating that increased serotonin levels may be involved with enhanced learning (Kim et al. 1997). Additionally, 5-HT_{1A} receptor knockout mice have changes in learning and memory associated with decreased serotonin release and revealed by a deficit in hippocampal-dependent learning and memory tasks (Sarnyai et al. 2000). Evidence also indicates that serotonin re-uptake inhibitors enhance learning and memory (Meneses and Hong 1997).

One factor that can influence learning is attention (Gould and Wehner 1999). Work by Carli and Samanin using an agonist specific for the 5-HT_{1A} receptor indicated that activation of the 5-HT_{1A} autoreceptor decreased release of serotonin and reduced accuracy in a serial reaction time task (Carli and Samanin 2000). The authors suggest that animals with impaired serotonin neurotransmission are unable to ignore stimuli that are irrelevant to the conditions of reinforcement in the serial reaction time task, resulting

in increased impulsivity and attentional dysfunction. Decreased serotonergic functioning might allow innocuous sensory signals to be processed through the amygdala as emotionally stimulating events (Stutzmann and LeDoux 2000). However, the receptors for serotonin that function postsynaptically to focus attention and reduce impulsivity in the presence of serotonin have yet to be determined. While it is established that serotonin plays a role in learning and memory and attention, the role of the 5-HT_3 receptor in learning and memory is less well understood.

Data strongly suggest that antagonism of the 5-HT_3 receptor improves learning and memory in associative tasks (Hong and Meneses 1996; Meneses and Hong 1997; Reeta et al. 1999; Rodgers et al. 1995; Roychoudhury and Kulkarni 1997). Autoshaping, an associative test involving lever pressing, was used to assess learning and memory in rats in response to antagonists or agonists of the 5-HT_3 receptor (Hong and Meneses 1996). The authors determined that, while antagonism of the 5-HT_3 receptor enhanced the retention of conditioned responding, drug effects could be ascribed to presynaptic autoreceptors, and not likely postsynaptic mechanisms. While studies suggest that blockade of the 5-HT_3 receptor improves learning, most studies that utilize the 5-HT_3 receptor antagonist ondansetron are often designed to reverse a scopolamine-induced deficit in learning (Barnes et al. 1990b; Carli et al. 1997; Ohno and Watanabe 1997; Roychoudhury and Kulkarni 1997). Ondansetron by itself has been found to have no effect on spatial learning or working memory in rats (Carli et al. 1997, Ohno and Watanabe 1997). Studies indicate that 5-HT_3 receptors influence learning and memory, yet the role of the endogenous receptor, independent of drug manipulation, has yet to be determined. Finally, it is an important observation that no

studies have reported effects of agonists or antagonists to the 5-HT_3 receptor in classical fear conditioning paradigms.

Distribution of the 5-HT_3 receptor is relatively ubiquitous, however the paucity of expression has made this receptor difficult to study *in vivo*. To overcome this difficulty, our laboratory has developed a transgenic mouse over-expressing the 5-HT_{3A} receptor in the forebrain on a B6xSJL/F1 background (5-HT_3-OE mice) (Engel et al. 1998). Data from electrophysiological and binding studies indicate that transgenic 5-HT_3 receptors are functionally indistinguishable from endogenous receptors and suggest that the transgenic receptors form functional ion channels (Engel et al. 1998; Sung et al. 2000). 5-HT_3-OE mice provide a valuable model for studying *in vivo* effects of this receptor. The natural distribution of the endogenous receptor in corticolimbic structures such as the amygdala and hippocampus lends this over-expressing animal model to studies of learning and memory (Doucet et al. 2000, Morales and Bloom 1997). In rats, 5-HT_3 receptors are densely labeled in the amygdala, neocortex and hippocampus, and in 5-HT_3-OE mice there is a clear increase of 5-HT_3 receptor expression in these areas determined by autoradiography (Engel et al. 1998). As we have limited expression of the 5-HT_3 receptor transgene to the forebrain and thus excluded it from neurons in the raphe nuclei that would express the transgene presynaptically as an autoreceptor, we are provided a unique opportunity to assess the behaviors of 5-HT_3-OE mice that are impacted by postsynaptic effects of receptor over-expression in the forebrain. Because the hippocampus and amygdala, particularly, are structures known to be involved in conditioned fear, a paradigm that can be used to assess learning and memory (Phillips and LeDoux 1992; Kim and Fanselow 1992; Maren 2001), studies were carried out

utilizing conditioned fear paradigms to assess learning and attention in 5-HT_3-OE and WT mice. In the present study we report the impact of 5-HT_3 receptor over-expression on learning, memory and attention, as well as related behaviors such as sensorimotor gating, anxiety, and exploratory behavior in response to novelty.

Results

5-HT_3-OE mice have enhanced contextual fear conditioning

Figure 1 presents the contextual and cued fear conditioning results for mice trained with a paired tone-shock (PTS) and mice trained with an immediate shock unpaired from the tone (IS). A three-way ANOVA (training x genotype x gender) indicated the effect of gender for the tone test and the context test was not significant, so data was collapsed across sex. A two-way ANOVA for the context test revealed a significant effect of genotype, $F(1,28)=31.2$, $p<0.001$ and a significant effect of training condition, $F(1,28)=244.6$, $p<0.001$. A significant interaction between genotype and training condition was also found in the context test, $F(1,28)=31.2$, $p<0.001$ indicating that the enhanced contextual conditioning seen in the 5-HT_3-OE PTS group was not seen in the 5-HT_3-OE IS group. However, the effect of genotype was not significant for the tone test. A significant effect of training condition was found for the tone test, $F(1,32)=122.5$, $p<0.001$.

Extinction is not different between WT and 5-HT_3-OE mice

Figure 2 illustrates the extinction rates to the context and tone for 5-HT_3-OE and WT mice. 5-HT_3-OE mice do not extinguish to the context or the tone at a faster rate

than WT mice, as there was no significant effect of genotype using a two-way ANOVA (genotype x day). A significant effect of day was found for both context and tone tests, $F(6,70)=234.8$, $p<0.001$ and $F(6,70)=35.2$, $p<0.001$, respectively. The rates of extinction indicate that 5-HT_3-OE mice do not have enhanced contextual extinction when compared to WT mice despite the enhanced contextual conditioning seen with fear conditioning.

5-HT_3-OE mice are less anxious in the elevated plus-maze

Figure 3 illustrates that 5-HT_3-OE mice are less anxious in the elevated plus-maze as reflected by the increased number of entries into the open arms, increased time spent in the open arms of the maze and decreased time spent in the closed arms. Time spent in the open center area was also increased relative to WT mice indicating a decrease in anxious behaviors observed for 5-HT_3-OE mice. All differences were significant ($p<0.02$) using unpaired t-tests. The concomitant decrease in the time spent in the closed arms of the maze indicates the increase in time spent in the open arms is not due to a generalized difference in locomotor activity between WT and 5-HT_3-OE mice.

Pain sensitivity is not different between WT and 5-HT_3-OE mice

Table 1 shows the three behavioral responses assessed for increasing shock intensities for both 5-HT_3-OE and WT mice. There was no significant difference in reactivity to footshock due to genotype. However, a sex difference was noted,

$F(1,60)=17.5$, $p<0.001$, as was a significant effect of the behavior being assessed, $F(2,60)=17.2$, $p<0.001$.

5-HT_3-OE mice have enhanced latent inhibition

Data for both context and tone tests were collapsed across sex as no main effect of gender was found. 5-HT_3-OE mice have enhanced latent inhibition to the tone compared to WT mice, as shown in Figure 4A. A significant effect of genotype was found using a two-way ANOVA (pre-exposure x genotype) for freezing to the tone, $F(1,54)=7.1$, $p=0.01$, as was an effect of pre-exposure, $F(2,54)=7.4$, $p=0.001$, and an interaction between genotype and pre-exposure, $F(2,54)=4.2$, $p=0.019$, reflecting the fact that context-tone pre-exposed 5-HT_3-OE mice have reduced freezing to the tone. A two-way ANOVA (pre-exposure x genotype) for freezing to the context revealed a main effect of genotype, $F(1,51)=7.7$, $p=0.008$, indicating that 5-HT_3-OE mice have enhanced contextual conditioning in this latent inhibition paradigm similar to the enhancement seen in fear conditioning (shown in Figure 4B).

PPI is not different between WT and 5-HT_3-OE mice

5-HT_3-OE mice are not different from WT mice in either PPI or maximum startle amplitude (Figures 5A and B). Data were collapsed across sex as no significant effect of gender was detected for PPI or startle. No significant effect of genotype on PPI was found using a two-way ANOVA (genotype x prepulse). A significant effect of prepulse intensity was detected, $F(3,72)=38.5$, $p<0.001$, indicating that increased prepulse intensity led to increased inhibition of the startle response for both transgenic and WT

mice. Transgene presence had no significant affect on the maximum startle amplitude. Results suggest that over-expression of the 5-HT_3 receptor has not altered sensory motor gating or the startle response.

5-HT_3-OE mice have enhanced exploratory behavior in response to novelty

Latency to leave the center during the initial 5 minute segment and to approach a novel object in the second segment is illustrated in Figure 6A. Exploratory behavior within the testing environment (initial 5 minute segment) and exploratory behavior in response to the novel object (second five minute segment with novel object – initial segment without novel object) is displayed in Figure 6B, 6C and 6D. No significant effect of gender was detected for the dependent variables of latency, time in center, number of center lines crossed, of transitions measured for either the initial or second segment. Data were collapsed across sex for all measures. Genotype did not significantly affect the latency to leave the center after placement in the open field. Similarly, latency to approach the novel object in the second segment of the test did not differ significantly, indicating that locomotor activity was similar in terms of latency for both genotypes in both segments. Time spent in the center during the initial five-minute segment did not differ significantly between WT and 5-HT_3-OE mice. A significant effect of genotype was found for the difference score of time spent in the center during the second segment – time spent in the center during the initial segment, $F(1,28)=22.0$ $p<0.001$, indicating that 5-HT_3-OE mice spend more time in the center area than WT mice when the novel object is present relative to behavior in the absence of the novel object. While genotype did not significantly affect the number of center line crossings

during the initial segment, an effect of genotype was significant for the measure of the difference in center line crossings between the second and first segments, reflecting the observation that 5-HT_3-OE mice displayed increased line crossings in the center during the second test segment compared to WT mice, $F(1,29)=15.0$, $p=0.001$. Total transitions during the initial test segment did not differ significantly between WT and 5-HT_3-OE mice, indicating that locomotor activity was similar between the two groups. However, a significant effect of genotype was found for the difference score between the first and second segment, $F(1,29)=5.0$ $p=0.033$. 5-HT_3-OE mice demonstrate enhanced exploratory activity in response to insertion of the novel object.

Discussion

Behavioral responses of 5-HT_3-OE mice were markedly different from WT mice in both contextual fear conditioning and latent inhibition tasks assessing learning and attention. PTS-trained 5-HT_3-OE mice displayed enhanced conditioning to contextual cues compared to WT, while auditory conditioning remained at similar levels in conditioned fear PTS groups. Administration of footshock at the beginning of the exposure to the context is known to cause a freezing deficit in subsequent tests (Lattal and Abel 2001a), and, as seen in Figure 1, the IS groups had deficits in freezing compared to PTS groups. No differences between genotypes were noted for IS groups in either the context or tone tests, indicating that conditions which produce a deficit in association formation and cause low levels of freezing are not impacted by over-expression of the 5-HT_3 receptor. Furthermore, the increase in contextual conditioning for PTS-trained 5-HT_3- OE mice was evident in the context test portion of the latent

inhibition paradigm revealing that contextual conditioning was enhanced in two different learning paradigms with differing parameters. The reduction in freezing levels in the LI context test compared to the fear conditioning test is likely due to differences in the time spent within the actual context on training days. The LI protocol allowed for 50 seconds less time during training in the context. Based on the PTS tone test responses in the fear conditioning paradigm, it is unlikely that 5-HT_3-OE mice are simply better at performing the conditioned response of freezing or are more sensitive to the footshock used for training than WT mice. However, previous studies have indicated that increased serotonergic neurotransmission in MAO-A deficient mice might be related to elevated pain thresholds in these animals (Kim et al. 1997). Because of the possible link between nociception and receptors for serotonin, mice were assessed to ensure that pain sensitivity was not changed in transgenic animals. 5-HT_3-OE mice were not different from WT in 3 measures of sensitivity to the footshock, as supported by the lack of a difference in the tone tests for conditioned fear. While there was an effect of gender, this was likely due to weight differences among male and female mice in both groups (data not shown). Thus, differences in pain sensitivity could not account for the improvements noted in contextual conditioning.

Based on the observation that response to the auditory cue was unchanged in 5-HT_3-OE compared to WT mice while contextual conditioning was enhanced, we conclude that hippocampal-dependent learning processes were affected by transgene expression. Lesion studies have shown that, while the amygdala is necessary for both contextual and cued conditioning, only contextual conditioning requires the hippocampal formation (Phillips and LeDoux 1992; Kim and Fanselow 1992). The hippocampus

possesses a large number of 5-HT_3 receptor expressing neurons and is necessary not simply for learning, but for discriminating; determining when it is appropriate to learn one thing in relation to another. Thus, an important role of the hippocampus is to inhibit unnecessary associations and allow for the formation of meaningful or predictive associations (Corcoran and Maren 2001; Wall and Messier 2001). 5-HT_3 receptor expression is increased in hippocampal GABAergic interneurons as well as principal excitatory neurons of 5-HT_3-OE mice (written communication, M. Morales 2001). Because serotonin is released preferentially in the hippocampus in response to stressful or fearful situations, 5-HT_3 receptor activation has the potential to connect emotionally salient events to hippocampal memory systems (Inoue et al. 1996; Wilkinson et al. 1996). The over-expressed receptor may, in some manner, contribute to hippocampal functioning and learning and memory by enhancing the contrast between relevant (emotionally salient) and irrelevant stimuli in learning paradigms.

Extinction to the auditory cue and to the context was unaffected by over-expression of the 5-HT_3 receptor. WT and 5-HT_3-OE mice were able to learn that the tone and the context were no longer predictive of the aversive footshock at similar rates. As extinction in the context is context specific and thus depends on the hippocampus (Corcoran and Maren 2001), 5-HT_3-OE mice do not appear to display hippocampal-dependent learning differences in extinction paradigms. This protocol indicates that 5-HT_3-OE animals have enhanced hippocampal-dependent learning and memory that depends on the novelty of the learning situation and cues. Extinction is an active learning process that does not erase, but rather suppresses, the original learning. Similar to the original fear conditioning, extinction requires acquisition, consolidation and

retrieval of new memories (Abel and Lattal 2001; Berman and Dudai 2001). The main difference between conditioned fear experiments and extinction experiments is that the situation is completely new, and therefore novel, in the original conditioned fear experiments. The absence of novelty in the extinction protocol and the lack of extinction enhancement for 5-HT_3-OE mice indicate that the enhancement of the context-footshock association may depend on the novelty of the learning paradigm (Abel and Lattal 2001; Lattal and Abel 2001b). The 5-HT_3 receptor may be important in the distinction between learning something new about a previous association and learning something completely new and therefore novel. Additionally, the novelty of the learning paradigm may enhance the saliency of emotionally relevant cues for 5-HT_3-OE mice compared to WT mice.

5-HT_3-OE mice are less anxious in the elevated plus-maze as indicated by the increased amount of time spent on, and number of transitions out to, open arms of the maze compared to WT mice. An anxiolytic effect of over-expression of the 5-HT_3 receptor is further supported by the fact that 5-HT_3-OE mice spent less time in the closed arms and more time in the open center compared to WT mice. Using an ethological version of the elevated plus-maze, Rodgers *et al.* determined that anxiogenesis was indicated for a low concentration of the 5-HT_3 receptor antagonist ondansetron (Rodgers et al. 1995). Other studies investigating the role of the 5-HT_3 receptor in fear/anxiety have reported conflicting results, perhaps due to differences in paradigms and drug dosages (Gonzalez et al. 1998; Nevins and Anthony 1994, Yoshioka et al. 1995). Here we report that over-expession of the 5-HT_3 receptor is anxiolytic in the both the elevated plus-maze and novelty exploration. Reduced anxiety

may contribute to the enhanced learning and memory in contextual fear paradigms noted for 5-HT_3-OE mice.

5-HT_3-OE mice react to the presentation of a novel object with increased inspective and inquisitive behavior. Inspective behavior is enhanced in 5-HT_3-OE mice as reflected by increased time in the center near the novel object compared to WT mice. The increase in number of entries into the area of a novel object also indicates increased inquisitive behavior (Grailhe et al. 1999; Dellu et al 2000) in 5-HT_3-OE mice compared to WT mice. Both indices support the idea that behavioral activation in response to novelty is significantly enhanced for 5-HT_3-OE mice compared to WT mice, and this may be the reason that 5-HT_3-OE mice display enhanced contextual conditioning compared to WT mice, yet do not have enhanced extinction. It could be argued that 5-HT_3-OE mice have increased locomotor activity regardless of the presence of a novel object; however, 5-HT_3-OE mice are not different from WT during the first 5 minutes in terms of total transitions, and 5-HT_3-OE mice do not have heightened basal open field activity indicating that the behavioral activation seen in this paradigm reflects a reaction to novelty and not increased locomotor activity (Engel and Allan 1999).

Decreased anxiety, specifically if 5-HT_3-OE mice are less anxious during the conditioned fear training, may contribute to enhanced hippocampal-dependent learning. While fearful reactions are generally considered to be controlled by the amygdala (Davis and Whalen 2001;Rogan and LeDoux 1996), the hippocampal formation also is important in anxious behaviors (Crestani et al. 1999; Gonzalez et al. 1998; McNaughton and Gray 2000). In fact, the hippocampal formation is thought to be capable of coding

critical aspects of anxiety, allowing for the integration of anxiety states and learning cues. Serotonin has long been known to play a role in anxiety and is preferentially released into the hippocampus during conditioned fear stress (Inoue et al. 1996; Wilkinson et al. 1996). Enhanced hippocampal-dependent learning seen in 5-HT_3-OE mice may be linked with reduced anxiety while in a novel situation that causes release of serotonin into the hippocampus, and therefore an enhanced ability to discriminate between predictive and irrelevant stimuli.

LI tasks are thought to reflect decremental attention, and so the less able the animal is to use a cue that was previously learned to be irrelevant, the better the latent inhibition (Gould and Wehner 1999). As opposed to incremental attention, where an animal learns to pay attention to relevant stimuli, decremental attention is the ability to ignore irrelevant stimuli. 5-HT_3-OE mice learned to ignore the irrelevant tone (of low predictive value) during pre-exposure and thus were less able to use the stimuli as an associative cue the next day. WT mice didn't appear to have learned anything about the relevance of the stimuli on the pre-exposure day and displayed poor LI. Previous studies indicate that antagonism of the 5-HT_3 receptor facilitates LI (Moran and Moser 1992). This reflects an enhancement in decremental attention with blockade of the 5-HT_3 receptor (Moser et al. 2000), yet current results indicate that 5-HT_3-OE mice have improvements in decremental attention when more receptor is available to bind serotonin, as is the case with over-expression of the 5-HT_3 receptor. The apparent discrepancy between our results and those previously reported may be due to differences in the LI paradigms used as well as species differences between rat and mouse (Moran and Moser 1992). Here we report the enhancement of LI as an effect of

chronic 5-HT_3 receptor transgene over-expression, which may be very different from acute effects of agonists or antagonists of this receptor.

PPI is thought to reflect a different kind of attention. PPI is a measure of the ability to shut out stimuli that quickly follow a previous stimulus and allows for mental integration (Dulawa and Geyer 2000). This mechanism is thought to be important in order to prevent the interruption of ongoing information processing routines by ensuing stimuli. Serotonin acts as a modulatory neurotransmitter in PPI paradigms (Caldarone et al. 2000; Dulawa and Geyer 2000; Paylor and Crawley 1997), so assessment of 5-H_3-OE and WT mice in this paradigm was necessary in light of the LI results that indicate enhanced decremental attention to a stimulus. Sensorimotor gating, as reflected by PPI to a tactile startle stimulus, appears to be normal in 5-HT_3-OE mice and shows the characteristic dependence on prepulse intensity, as does WT PPI. Startle amplitude is also unaffected by transgene expression. Previous reports indicate that agonists of the 5-HT_{1A} receptor reduce serotonin release and enhance PPI, while MDMA, a serotonin releasing agent, decreases PPI (Dulawa and Geyer 2000). The over-expression of the 5-HT_3 receptor did not appear to influence PPI or the startle response.

It is becoming increasingly obvious that the 5- HT_3 receptor is a valuable receptor to target for drug development (Johnson et al. 2000; Reeta et al. 1999; Sirota et al. 2000, Ye et al. 2001), so it is necessary to understand the role of this receptor in behavior. The current results suggest that over-expression of the 5- HT_3 receptor results in decreased anxiety and subsequent or independent enhanced exploratory behavior in response to novel stimuli that may influence hippocampal-dependent learning and memory as well as attention. The 5- HT_3 receptor might contribute to hippocampal

functioning and learning and memory by enhancing the contrast between predictive and irrelevant stimuli in learning paradigms. The over-expression of the 5- HT_3 receptor is proposed to enhance the saliency of emotionally relevant novel cues.

Materials and Methods

ANIMALS

All of the procedures employed in the current studies were approved by the University of New Mexico Laboratory Animal Care and Use Committee. 5-HT_3-OE mice were developed by our laboratory and are described elsewhere (Engel et al. 1998). WT mice are transgene negative litter-mates of the 5-HT_3-OE mice. The genetic background of WT and 5-HT_3-OE mice is B6SJL/F2 (B6xSJL/F1 x B6xSJL/F1). Mice were housed 2-4 per cage in a room with a 12 hr light/dark cycle (lights on at 0800 hr). Standard chow and water were available *ad libitum*. Male and female mice, 60-120 days old, were used in the present experiments. Behavioral testing was conducted between 0800 and 1500 hr.

FEAR CONDITIONING

Fear conditioning took place in a Coulbourn® Habitest™ Modular Test System with two metal walls, two Plexiglas walls, and a stainless steel grid floor for administration of the footshock. The apparatus was located within a sound-attenuated chamber. 70% isopropanol was used to clean the walls and floor after the removal of each mouse from the training context. Conditioning protocols were adapted from those described by Paylor et al. (1994). Mice were assigned to two groups, paired tone shock

(PTS) or immediate shock (IS). The IS group was given an electric foot shock (0.6 mA, duration 2 seconds) within the first 5 seconds of being introduced into the conditioning context. After 3 minutes the tone conditioned stimulus (80 db, 6 Hz clicker) came on for 1 minute. 30 seconds after termination of the tone mice were removed from the conditioning context and returned to their home cage. PTS mice were trained as follows. After 90 seconds of habituation in the conditioning context, the tone came on. An electric footshock (0.6 mA) was delivered during the last two seconds of the 30 second tone. After another 90 seconds, the tone and footshock sequence was repeated. Thirty seconds after co-termination of the second tone and footshock sequence the animal was removed from the conditioning context and returned to its home cage. Both IS and PTS training took 4 ½ minutes each. Twenty-four hours later mice were reintroduced to the conditioning environment for the retention test. The conditioning context was again cleaned with 70% isopropanol between animals. The tone and the footshock were not delivered during this test session. The animal's behavior was observed and contextual conditioning was assessed using a time-sampling procedure. Every ten seconds the mouse was scored as either moving or freezing. Freezing is the conditioned response measured to reflect the amount of learning and is defined by the absence of movement other than that required for respiration (Paylor et al. 1994). Approximately 2 hours later, freezing to an altered context (in a clear plastic container with orange scented bedding) and to the tone in this altered context was assessed. The altered context was cleaned with 70% ethanol after each mouse was tested. Freezing was scored for 3 minutes without presentation of the tone to determine basal levels of freezing in the altered context. After basal scoring, the tone came on and remained on while freezing was

scored for three minutes. The amount of freezing is represented as the % freezing (# of freezing intervals ÷ total intervals).

A three-way (training x genotype x gender) analyses of variance (ANOVA) was used to analyze the context and tone test data.

EXTINCTION

5-HT_3-OE and WT mice were trained as described for fear conditioning. Two groups were assigned for extinction, one for extinction to the context and one for extinction to the tone. Freezing to the context was assessed once every 24 hours for the context extinction group and freezing to the tone was assessed once every 24 hours for the tone extinction group. The conditioning context was cleaned with 70% ethanol and the altered context with 70% isopropanol. When mice displayed basal levels of freezing the testing was halted.

A two-way ANOVA (day x genotype) was used to assess extinction to the context and to the tone. Data for each mouse was normalized to the level of freezing during the first test and expressed as a percentage of the first test.

ELEVATED PLUS-MAZE

Mice were placed in the center square (6 x 6 cm) of a Plexiglas maze shaped in a cross elevated 2 feet above the ground. The supports that elevate the maze were made of clear Plexiglas and were positioned in the middle of the arms so the mouse was unable to detect them. The maze had two open arms (30 x 6 cm each) and was located in a moderately lit, sound attenuated room. Open arms consisted of the clear

Plexiglas floor and no walls. The closed arms were covered with black contact paper and had walls 6 cm high also covered with black contact paper. Behavior was monitored for 5 minutes with the aid of a video camera. The percentage of time spent in the open arms, closed arms and center area as well as the number of entries (two front paws) into the open and closed arms was recorded.

Unpaired t-tests were used to assess the affect of genotype on anxious behaviors.

PAIN SENSITIVITY

Procedures were as previously described by Crawley (1999). Responses to the footshock were assessed after mice had been extinguished in the context. The intensity of shock required to elicit running, vocalization and a jump was determined for each mouse by delivering a 1 second shock every 30 seconds starting at 0.08 mA and increasing the shock by 0.02 mA each time. The maximum intensity tested was 0.20 mA.

A three-way ANOVA (measured response x genotype x gender) was used to analyze shock reactivity.

LATENT INHIBITION

Procedures were adapted from those previously described by Caldarone et al. (2000). WT and 5-HT_3-OE mice were separated into three groups and pre-exposed to the training context alone (context group), to the context with presentation of the tone (context-tone group), or received no pre-exposure of context or tone. The conditioning chamber was cleaned with 70% isopropanol after the removal of each mouse during

pre-exposure. Mice in the context-tone group received 40 presentations of a 5 second tone (80 db, 6 Hz clicker, intertrial interval 30 seconds) within the training context previously described for fear conditioning. Mice in the context group were placed in the training chamber for the same amount of time (23 minutes, 50 seconds) as the context-tone group but received no exposure to the tone. 24 hours after pre-exposure, mice from each pre-exposure condition and the no pre-exposure group were placed in the conditioning context for training. The apparatus was again cleaned with 70% isopropanol between animals. After 90 seconds of habituation the tone was presented for 5 seconds and a footshock (0.6 mA) was delivered through the stainless steel grid floor for the last two seconds of the tone. 30 seconds after the first footshock, the tone and footshock sequence were repeated and mice were removed from the context 30 seconds after co-termination of the 2nd tone/shock pairing. The day after training mice were observed for a freezing response to the tone. On the following day mice were scored for freezing to the original conditioning context (where both the pre-exposure and training had occurred).

A three-way ANOVA (pre-exposure type x genotype x gender) was used to assess freezing to the context and to the tone after training in the LI paradigm.

ACOUSTIC PREPULSE INHIBITON OF TACTILE STARTLE RESPONSE

Testing was conducted in the SR-Lab System (San Diego Instruments, San Diego, CA, USA) startle apparatus as previously described by Paylor and Crawley (1997). The background noise level of the chamber was adjusted to 70dB. The sound levels for the background noise and each auditory stimulus were calibrated using a

digital sound level meter (Radio Shack Sound Level Meter). Mice previously used for extinction testing were used in all PPI tests. They had been allowed 2 weeks between the last day of extinction testing and the PPI test. Each test session began with a 5 minute acclimation period in which the animal was left alone in the Plexiglas tube within the sound attenuated chamber with the 70 dB background noise and house light on. After the 5 minute acclimation period each subject was presented with 60 trials. Each test session consisted of 10 trial types. One trial was a 40-ms, 12 psi tactile (air puff to the back of the animal) startling stimulus. There were four different acoustic prepulse plus tactile stimulus trials presented so that the prepulse stimulus onset occurred 100-ms before the onset of the tactile stimulus. The prepulse stimuli were 20-ms sounds of 78, 82, 86, or 90 dB. There were also trials to assess startle to each prepulse intensity without a subsequent tactile stimulus. Each of the prepulse intensities was presented for 40-ms for these trials. Finally, there were trials with no stimulus to measure baseline movement within the cylinder. The 10 trial types were presented pseudorandomly so that each trial type was presented once within each of the 6 blocks of trials. The average intertrial interval (ITI) was 15 seconds and ranged from 10 to 20 seconds. The startle response was recorded for 65 ms (measurements taken every 1 ms) beginning at the stimulus onset. Maximum and average startle values for this time period were recorded. The maximum startle amplitude was used as the dependent variable. Percent prepulse inhibition of the startle response was calculated as 100-[(startle response on acoustic prepulse plus tactile stimulus trial/tactile startle response alone trial) x 100]. A high percent prepulse inhibition value reflects strong prepulse inhibition

indicated by a reduced startle response when a prepulse stimulus precedes the startling stimulus compared with when the startling stimulus is presented alone.

A three-way ANOVA (prepulse intensity x genotype x gender) was used to assess inhibition of the startle response in the presence of a prepulse. A two-way ANOVA (genotype x gender) was used to assess startle in the absence of the prepulse.

NOVEL OBJECT EXPLORATION

The test was adapted from the protocol previously described by Grailhe et al. (1999). An open field apparatus was used measuring 17" x 17" with 8" high Plexiglas side-walls. The floor of the apparatus was black and divided up into 5 areas: 4 equal quadrants and 1 center area with a diameter of 14 cm. The experiments were carried out in a dimly lit room with the aid of a video camera to minimize any effects of stress/anxiety. The floor and walls were wiped with 70% isopropanol before each test session. The test session consisted of two 5 minute periods. For the first five minutes, mice were placed into the center area and latency to leave the center was recorded, the time spent in the center was assessed, the total number of center line crosses and the number of total lines crossed was tallied. During the second five minutes the same measures were taken after introduction of a novel object into the center. Latency to approach the object also was recorded. The object was a pink and green striped gray cube (1 $inch^3$) with an open side. The cube was placed in the center with the open side facing the mouse.

A two-way ANOVA (genotype x gender) was used to analyze each of the four measures of reactivity to the novel environment (initial segment score, first five minutes) and to

analyze the same four measures of reactivity to the novel object using a difference score of the second five minute segment minus the initial five minute segment scores.

Acknowledgments

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Figure Legends

Figure 1. 5-HT_3-OE mice have enhanced contextual fear conditioning.

Data are mean ± SEM. (v) 5-HT_3-OE, (v) WT. N = 10 for PTS tone test, N = 8 for PTS context test and 8 for both IS test groups. Triple Asterisk indicates that PTS trained 5-HT_3-OE mice froze significantly more to the context than WT mice, $p<0.001$.

Figure 2. 5-HT_3-OE mice and WT mice do not differ in extinction.

Data are mean ± SEM. (v) 5-HT_3-OE, (v) WT. N=6 for both 5-HT_3OE and WT. A) Context test. B) Tone test. Data for each mouse was normalized to the level of freezing during the first test and expressed as a percentage of the first test.

Figure 3. 5-HT_3-OE mice are less anxious in the Elevated Plus-Maze.

Data are mean ± SEM. (v) 5-HT_3-OE, (v) WT. N = 6 for both 5-HT_3OE and WT. Asterisk indicates a significant difference at $p<0.02$ by unpaired t-test. A) Number of entries into the open arms expressed as a percentage of total arm entries. B) Time in seconds spent in the open arms of the maze. C) Time in seconds spent in the closed arms of the maze. D) Time in seconds spent in the center of the maze.

Figure 4. Latent Inhibition to the tone is enhanced in 5-HT_3-OE mice while contextual conditioning remains elevated.

Data are mean ± SEM. (v) 5-HT_3-OE, (v) WT. A) Conditioned responding to the tone. N=8 for no pre-exposure groups and 11 for context and context-tone groups. 5-HT_3-OE mice have improved latent Inhibition relative to WT mice as shown by decreased freezing in the context-tone pre-exposed group relative to context only pre-exposed group. The asterisk indicates a significant difference at $p=0.02$. B) Conditioned responding to the context. N=7 for 5-HT_3-OE no pre-exposure, N=9 for WT no pre-

exposure, N=8 for 5-HT_3-OE context pre-exposure, N=9 WT context pre-exposure, and N=12 for context-tone pre-exposure groups. ANOVA indicated a significant effect of genotype indicating that contextual conditioning was enhanced for 5-HT_3-OE mice relative to WT regardless of pre-exposure conditions (p=0.008).

Figure 5. 5-HT_3-OE mice do not have altered pre-pulse inhibition or startle.

Data are mean ± SEM. (ν) 5-HT_3-OE, (▽) WT. N = 10 for both. A) PPI. X-axis labels indicate the intensity of the prepulse in decibels. B) Max startle amplitudes. Y-axis indicates numerical value given for the force detected on the floor of the startle apparatus in response to stimuli; the larger the number, the greater the startle.

Figure 6. 5-HT_3-OE mice have increased exploratory behavior in response to a novel object.

Data are mean ± SEM. (ν) 5-HT_3-OE, (▽) WT. Exit reflects the latency to leave the center after placement in the open field. Approach indicates the latency to approach the novel object. Initial bars indicate behavior in the first five minutes. Difference bars reflect the difference score between the first five minute and second five minute segments (segment 2 – segment 1). **A)** Latency, 5-HT_3-OE N=13, WT N=16. **B)** Time in center, 5-HT_3-OE N=13, WT N=17. Triple asterisk indicates p<0.001. **C)** Number of times center lines were crossed, 5-HT_3-OE N=13, WT N=18. Double asterisk indicates p=0.001. **D)** Total lines crossed, 5-HT_3-OE N=13, WT N=18. Asterisk indicates p=0.033.

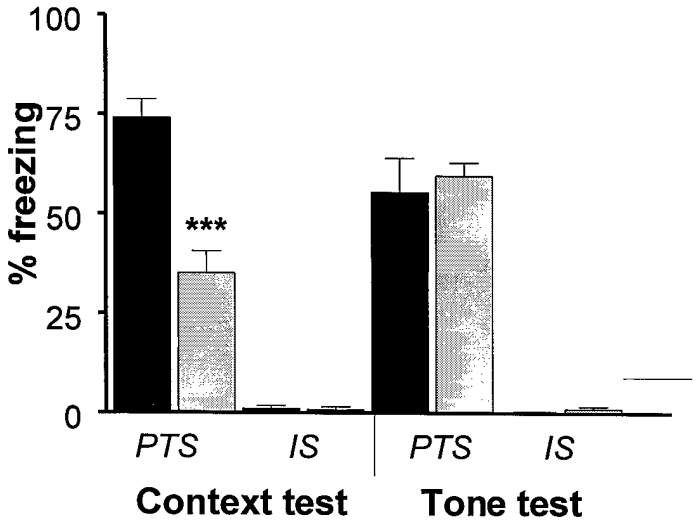
Tables

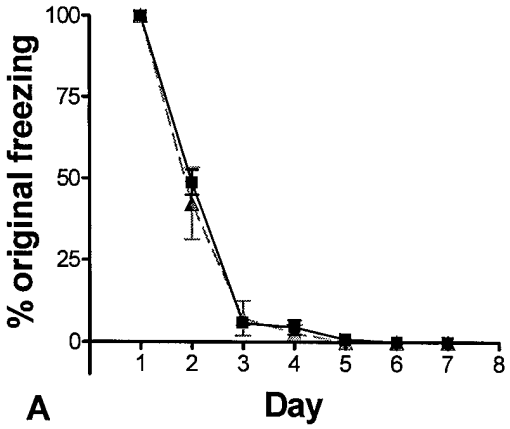
Table 1. Pain/sensitivity thresholds are not different

Numbers are in mAmps. Data are mean ± SEM. N= 12.

	Movement	Vocalization	Jump
5-HT_3-OE	0.09 ± 0.004	0.12 ± 0.01	0.14 ± 0.01
WT	0.09 ± 0.004	0.11 ± 0.01	0.14 ± 0.01

Figures

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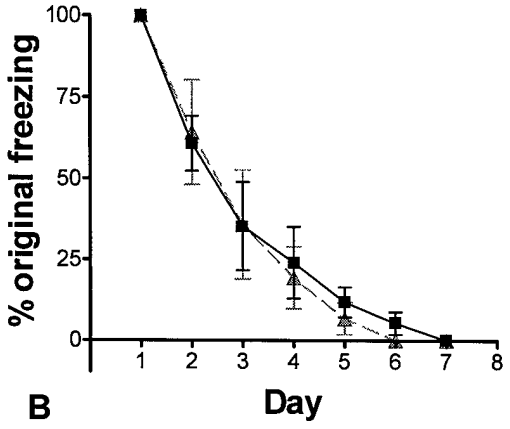
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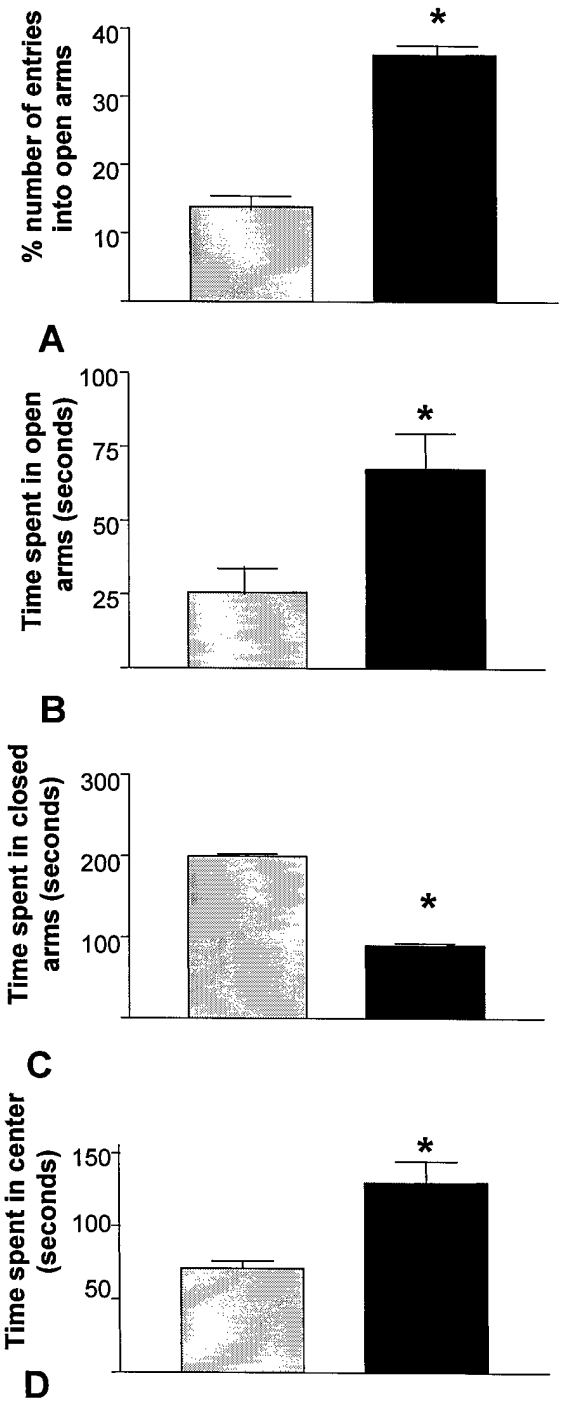
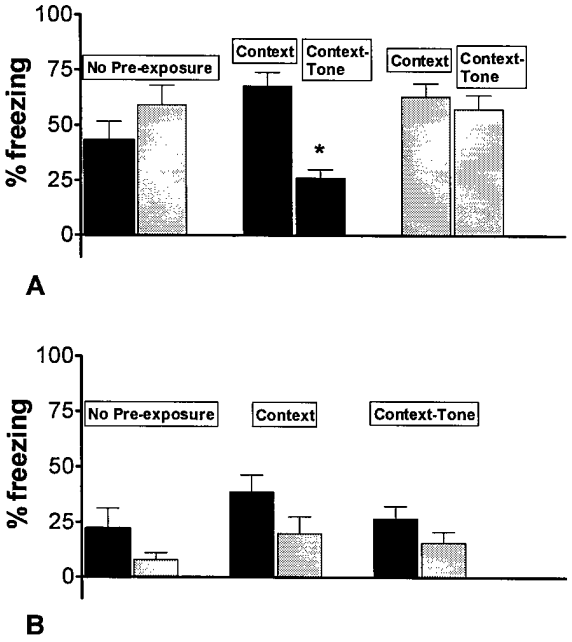

% number of entries into open arms
40
30
20
10
*
A
Time spent in open arms (seconds)
100
75
50
25
*
B
Time spent in closed arms (seconds)
300
200
100
*
C
Time spent in center (seconds)
150
100
50
*
D


100
75
50
25
0
% freezing
No Pre-exposure
Context
Context-Tone
Context
Context-Tone
*
A
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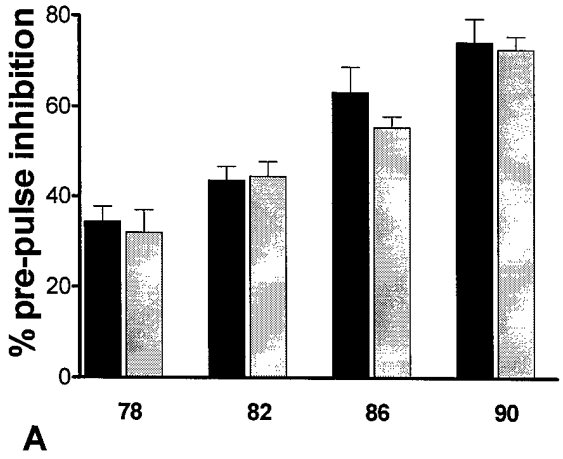
% freezing
No Pre-exposure
Context
Context-Tone
B


% pre-pulse inhibition
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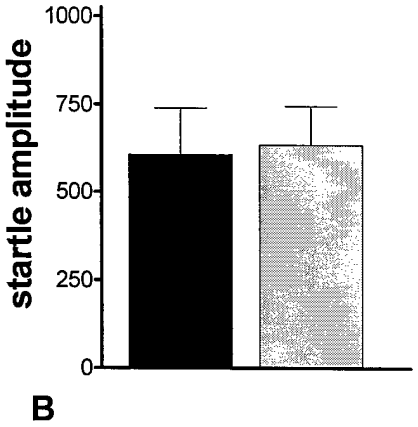
60
40
20
0
78
82
86
90
A


startle amplitude
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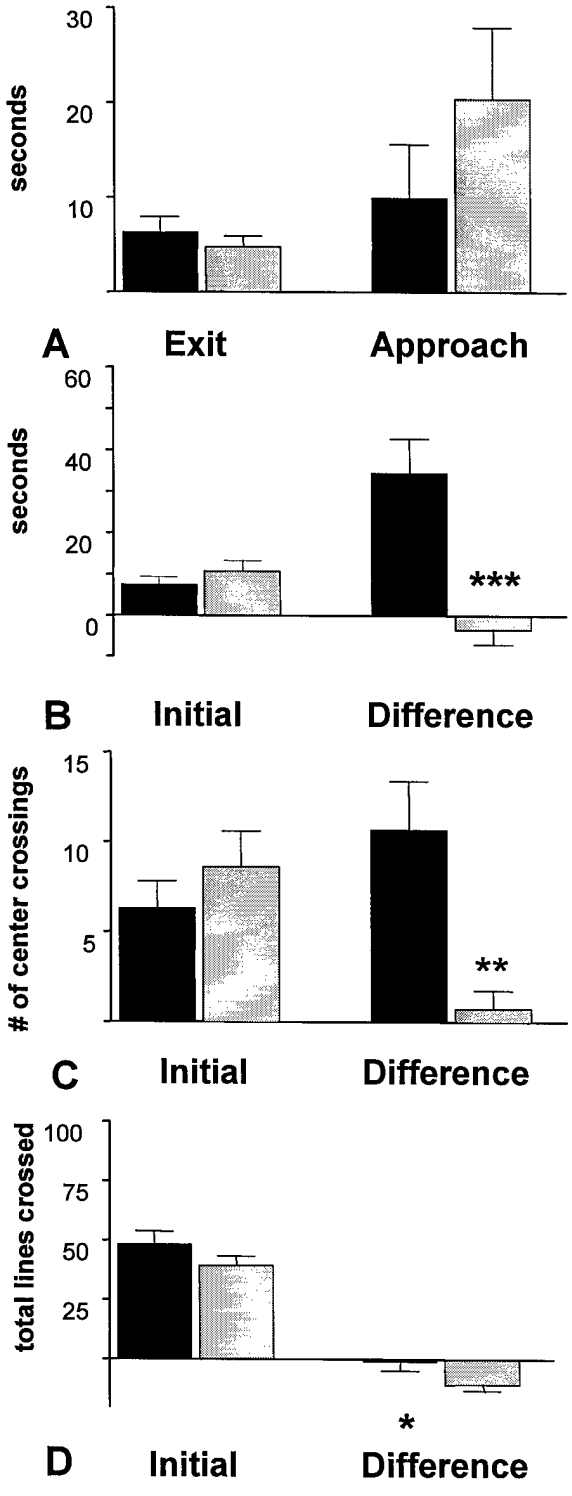
750
500
250
0
B


30
20
10
seconds
A
Exit
Approach
60
40
20
0
seconds
***
B
Initial
Difference
15
10
5
# of center crossings
**
C
Initial
Difference
100
75
50
25
total lines crossed
*
D
Initial
Difference

ALLAN Appendix

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A mouse model of prenatal ethanol exposure using a voluntary drinking paradigm.

Andrea M. Allan, Julie Chynoweth, Lani A. Tyler, and Kevin K. Caldwell

Department of Neurosciences, University of New Mexico Health Sciences Center, Albuquerque, New Mexico, 87131-0001.

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Running Title: Learning deficits in FAE mice

Corresponding Author:

Andrea M. Allan, Ph.D.

Department of Neurosciences MSC 08-4740

1 University of New Mexico

Albuquerque, New Mexico 87131-0001

Telephone: 505-272-8811

Fax: 505-272-8082

E-mail: aallan@salud.unm.edu

ABSTRACT

Background: The incidence of fetal alcohol spectrum disorders (FASD) is estimated to be as high as 1 in 100 births (Sampson et al. 1997). Efforts to better understand the basis of prenatal ethanol-induced impairments in brain functioning, and the mechanism(s) by which ethanol produces these defects, will rely on the use of animal models of fetal alcohol exposure (FAE).

Methods: Using a saccharin sweetened alcohol solution, we developed a free choice, moderate alcohol access model of prenatal alcohol exposure. Stable drinking of a saccharin solution (0.066%) was established in female mice. Ethanol was then added to the saccharin in increasing concentrations (2%, 5%, 10% w/v) every two days. Water was always available and mice consumed standard pellet chow. Control mice drank saccharin solution without ethanol. After a stable baseline of ethanol consumption (14 g/kg/day) was obtained, females were impregnated. Ethanol consumption continued throughout pregnancy, then was decreased to zero percent in a step-wise fashion over a period of six days after pups were delivered. Characterization of the model included measurements of maternal drinking patterns, blood alcohol levels, food consumption, litter size, pup weight, pup retrieval times for the dams and effects of FAE on performance in fear-conditioned learning and novelty exploration.

Results: Maternal food consumption, maternal care, and litter size and number were all found to be similar for the alcohol-exposed and saccharin control animals. FAE did not alter locomotor activity in an open field, but did increase the time spent inspecting a novel object introduced into the open field. FAE mice displayed reduced contextual fear, when trained using a delay fear conditioning procedure.

Conclusions: The mouse model should prove to be a useful tool in testing hypotheses about the neural mechanisms underlying the learning deficits present in FASD. Moreover, a mouse prenatal ethanol model should increase the opportunity to use the power of genetically defined and genetically altered mouse populations.

Key words: fetal alcohol syndrome, ethanol, mouse, voluntary drinking behavior

The incidence of fetal alcohol spectrum disorders (FASD), which includes Fetal Alcohol Syndrome (FAS) and alcohol-related neurodevelopmental disorder (ARND) (Streissguth and O'Malley, 2000), is estimated to be as high as 1 in 100 births (Sampson et al., 1997). Children with FASD display a variety of cognitive and behavioral aberrations, ranging from severe mental retardation to subtle deficits that become apparent under stressful conditions (Streissguth et al., 1990; Streissguth et al., 1994; Mattson and Riley, 1998). Efforts to better understand the basis of prenatal ethanol-induced impairments in brain functioning, and the mechanism(s) by which ethanol produces these defects, will rely on the use of animal models of fetal alcohol exposure (FAE).

Most studies on fetal alcohol effects have employed the rat using a variety of ethanol administration paradigms and schedules. Prenatal ethanol exposure paradigms commonly either require dams to ingest an ethanol-containing liquid diet or to be intubated, or injected, with ethanol. These procedures produce variable degrees of maternal stress, which, in turn, may modify the effects of prenatal drug exposure on the developing fetus (Ward and Wainwright, 1989; Slone and Redei, 2002). The use of liquid diets also limits the amount of ethanol that can administered without inducing malnutrition. An additional confound associated with many existing paradigms of fetal alcohol exposure (FAE) is that cross-fostering of the pups is employed in order to avoid the introduction of differences in maternal care that may occur between control (non-alcohol consuming) and alcohol drinking moms, whether alcohol availability is continued postpartum, or whether alcohol is withdrawn. The quality of maternal care has been shown to produce significant changes in hippocampal synaptophysin immunoreactivity and Morris water maze performance in the offspring (Liu et al., 2000). Even brief maternal separation (45 min) has been shown to increase the expression of nerve growth factor in the dentate gyrus and hilus regions of the hippocampal (Cirulli et al., 1998).

We sought to develop a mouse model of FAE that minimized these problems. In the present studies, we used saccharin sweetened ethanol solutions (Czachowski et al., 1999; Roberts et al., 1999; Tomie et al., 2002) and a modification of the sucrose fading approach (Slawecki et al., 1997) to establish consistent, voluntary alcohol consumption patterns prior to impregnating female mice, then maintained alcohol consumption throughout gestation. Nutrition was provided using a standard pellet diet. Following delivery of the offspring, step wise decreases in ethanol concentrations in the saccharin solution prevented the display of ethanol withdrawal signs in the mother and eliminated the need to cross-foster the pups. This procedure did not significantly impact various measures of maternal or neonatal well-being, or maternal care for the pups, yet resulted in cognitive and behavioral impairments in the offspring consistent with other models of FAE.

MATERIALS AND METHODS

Ethanol Drinking and Fetal Alcohol Exposure Paradigms.

All of the procedures employed in the current studies were approved by the University of New Mexico Health Sciences Center Laboratory Animal Care and Use Committee.

Sixty day-old B6SJL/F1 (Harlan Industries, Indianapolis, IN) female mice were individually housed in plastic cages in a temperature controlled room (22 °C) on a 12 hr dark: 12 hr light schedule (lights on from 0700 to 1900 hr). Standard chow and water were available ad libitum.

Prenatal exposure of mice to ethanol was performed using saccharin sweetened solutions (Czachowski et al., 1999; Roberts et al., 1999; Tomie et al., 2002). Female mice were offered 22 hr free access to either 0.066% saccharin or water for 2 weeks. Subsequently, ethanol was added to the saccharin tube for the experimental groups, while the control group continued to have access to saccharin alone. The concentration of ethanol was increased in a step wise fashion every two days, from 0% to 2%, to 5%, then, finally, to 10% (w/v). After two weeks of drinking, a male was introduced into the female's cage. Once the female was determined to be pregnant by the presence of vaginal plug, the male was removed and nesting material was placed in the cage. Ethanol consumption was not measured for the 1-2 days while the male was present in the cage. Females continued to drink stably throughout pregnancy. Within one day of birth, the alcohol and the saccharin concentrations were reduced by one-half every two days, until the mice were consuming only water. Saccharin consuming mothers were weaned off of the sweetened water in a similar step down fashion. Litter sizes and litter weights were determined on postnatal day (PND) 7. Pups were weaned at 23 days and maintained in same sex, litter-mate housed cages with free access to water and chow. Offspring (both male and

female) were 60-100 days old, when used in the present experiments. No more than two littermates were represented in any single treatment condition.

Characterization of Maternal Care

Maternal grooming (frequency and duration) and care for the pups (latency to retrieve pups removed from the nest and time spent on the nest) were evaluated at PND 3-5 between 1000 and 1300 hr by videotape monitoring.

Blood Ethanol Measurement

Maternal blood ethanol concentrations produced by ad libitum consumption of the 10% ethanol/0.066% saccharin solutions were measured using saphaneous vein puncture (a 10 μL sample) and determined enzymatically (Farr et al., 1988). Blood samples were collected from nine separate groups of mice at 15 different times between 0000 and 2400 hr during the second week of gestation; each dam gave a maximum of three different samples. Blood alcohol was determined for three separate breeding rounds of FAE animals. Since restraint and blood taking are stressful, these measures were performed on a separate group of dams who were drinking at a rate similar to the experimental dams; the pups from these dams were not used in these studies.

Whole blood was collected from the saphenous vein and immediately mixed with 0.2 mL of 6.6% perchloric acid and stored frozen at -20 °C until assayed. Blood ethanol standards were created by mixing whole blood from untreated mice with known amounts of ethanol ranging from 0 to 240 mg/dL and then mixing 0.1 mL aliquots of each standard with perchloric acid and storing the standards frozen with the samples. Blood ethanol samples were assayed using a

modification of the method of Lundquist (1959). The ethanol standard curve was linear over the 0-240 mg/dL range. Sample blood ethanol values were determined by regression analysis.

Corticosterone Radioimmunoassay and Adrenal Weights

After obtaining body weights, adult mice were rapidly decapitated, blood was collected, and the adrenal glands were dissected, separated from the capsule and wet weighed. Trunk blood was collected into chilled tubes containing EDTA (7.5 mg) and aprotonin (0.2 mL/0.5 mL blood) and centrifuged at 2,200 x g for 10 min at 4 °C. Supernatant (plasma) was then collected into clean tubes and stored at -80 °C until used in the assay. Corticosterone levels were analyzed in duplicate using a mouse corticosterone RIA kit (ICN Biochemicals, Costa Mesa, CA). The detection limit for the kit was determined to be 3 ng of corticosterone/mL with a within assay variance of 4.5% and a between assay variance of 6.5%.

Novel Object Exploration

The novel object test was adapted from the protocol previously described by Grailhe et al. (1999). An open field apparatus, measuring 17" x 17" with 8" high Plexiglas side-walls, was used. The floor of the apparatus was black and divided into five areas: four equal quadrants and one center area having a diameter of 14 cm. The experiments were carried out in a dimly lit room with the aid of a video camera to minimize effects of stress and anxiety. The floor and walls were wiped with 70% isopropanol before each test session. The test session consisted of two 5 minute periods. At the start of the first five-minute session, mice were placed into the center area and the latency to leave the center, the total time spent in the center and the total number of center line crosses and the number of total lines crossed were recorded as measures of exploration. At the start of the second five-minute period, a pink and green striped gray cube, one inch^3, with an open side was placed into the center of the

apparatus, with the open side facing the mouse. Measurements were made of the latency to approach the novel object, the total time spent in the center area, the total number of center line crosses and the number of total line crosses.

Delay Fear Conditioning

Fear conditioning was conducted using a procedure similar to that described by Wehner and colleagues (Lu and Wehner, 1997; Smith and Wehner, 2002). A Coulbourn Instruments (Allentown, PA) Habitest® System with two metal walls, two Plexiglas walls, and a stainless steel grid floor for administration of the foot shock was used for conditioning. The apparatus was located within a sound-attenuated chamber.

Mice were trained as follows. After 90 seconds of habituation in the conditioning context, the conditioned stimulus (CS), an 80 dB, 6 Hz clicker, was initiated. The unconditioned stimulus (US), an electric foot shock (0.55 mA), was delivered during the last two seconds of the 30 second CS. Ninety seconds later, the CS-US sequence was repeated. Thirty seconds after co-termination of the second CS-US pairing, the animal was removed from the conditioning context and returned to its home cage.

Approximately 24 hours later, mice were assessed for their conditioned responses to the context and to the CS by measuring freezing (defined by the absence of movement other than those necessary for respiration; Bouton & Bolles, 1980; Fanselow, 1980) behavior following reintroduction into the context in which conditioning occurred and exposure to the CS in a novel context (a clear plastic container with orange scented bedding). Freezing was measured using a time-sampling procedure in which every 10 seconds the mouse was scored as either moving or freezing. The amount of freezing (expressed as a percentage) was calculated by dividing the number of observations periods where the mouse was frozen by the total number of

observation periods. Freezing in the conditioning context (in the absence of the CS and US) was measured for four minutes. Freezing to the CS in the novel context was determined approximately one hour after assessment of contextual conditioning. Basal levels of freezing in the novel context were scored for three minutes without presentation of the CS. After basal scoring, the CS was initiated and remained on while freezing was scored for an additional three minutes.

RESULTS

Effect of saccharin fading ethanol exposure paradigm on food and fluid consumption.

We monitored the consumption of standard breeder chow and water as the ethanol was introduced. While there was a small increase in food consumption upon introduction of the saccharin (Figure 1A), there were no significant alterations in either the eating (Figure 1A) or drinking (Figure 1B) patterns of the mice. Figure 1B shows that the gradual introduction of ethanol was readily accepted by the mice, with a slight reduction in intake once the 10% (w/v) ethanol was introduced into the bottles. Saccharin drinking control moms maintained a stable level of preference for the saccharin across their pregnancy (Figure 2), drinking almost exclusively from the saccharin-sweetened water tubes. Similarly, the 10% (w/v) ethanol drinking moms developed a stable preference for the sweetened ethanol solution prior to pregnancy and maintained this preference throughout the pregnancy (Figure 2). It should be noted that, while there is a clear preference for the sweetened solutions, the mice reduced their water intake, such that there was no significant increase in their total fluid consumption.

Effect of saccharin fading ethanol exposure paradigm on maternal blood alcohol levels.

Blood alcohol levels, measured in a separate group of ethanol drinking dams were evaluated over a 24 hour period (Figure 3). Peak blood alcohol levels were achieved between 1600 hr and 0100 hr, during the dark cycle. The average daily consumption of ethanol by mouse dams on the 10% ethanol saccharin solutions was 14.0 g EtOH/kg body weight/day, generating average peak blood alcohol level of 120 ($\pm$ 9) mg/dL consistently throughout the pregnancy (Figure 3). The average calorie equivalent of the ethanol consumed was about 10% the daily diet intake.

Effect of saccharin fading ethanol exposure paradigm on pup weight, litter size and maternal weight.

Food consumption (Table 1) was monitored every other day and no difference between the saccharin sweetened ethanol and saccharin alone drinking females in average daily food consumption was noted. Additionally, there was no significant difference in the change of material weight from Day 1, the start of 10% (w/v) ethanol, to Day 17, the day male was introduced into the cage. Average pup weights and litter sizes did not significantly differ between the ethanol and saccharin drinking groups (Table1). No gross anatomical abnormalities were noted at birth in the fetal ethanol-exposed mice.

Effect of saccharin fading ethanol exposure paradigm on maternal grooming and latency to retrieve pups.

While pup weights and growth are good indicators of maternal care, we also addressed maternal care using a pup retrieval test, as well as monitoring grooming frequencies over 4 one-hour observation sessions (Table 2). The ethanol drinking moms retrieved pups that were deliberately removed from the nest with a similar latency to that of the saccharin drinking moms (Table 2). Further, ethanol consuming females did not differ from controls in the percent time spent on the nest or grooming of pups.

Effect of saccharin fading ethanol exposure paradigm on offspring basal corticosterone levels

Several studies have shown that rats prenatally exposed to ethanol demonstrate changes in hypothalamic-pituitary-adrenal responsiveness, as indicated by elevations of adrenocorticotropin and corticosterone, especially after stress (Glavas et al., 2001; Osborn et al, 1996). Levels of corticosterone in blood were determined without using any activating stress other than handling the mouse just prior to decapitation. In our study, FAE females had a

significantly higher plasma corticosterone level than did control females (Table 3). Although a similar difference was observed for males, it did not achieve significance. This was demonstrated by a significant interaction of sex x prenatal treatment condition interaction ($F(1,28)$ 5.67, $p<0.03$. No differences were observed in adrenal weights of FAE and control offspring.

Effect of saccharin fading ethanol exposure paradigm on novelty exploration.

Exploratory behavior within the testing environment (initial 5 minute segment, without object present) and exploratory behavior in response to introduction of a novel object (second five minute segment, with novel object) were assessed by recording the number of transitions, time spent in center and number of entries into the center area (Figure 4). In these studies adult male offspring were used. Data were analyzed by analysis of variance with Bonferroni correction. Prenatal condition (ethanol versus saccharin) did not significantly affect the number of entries into the center, regardless of the object presence (Figure 4,B); thus, this dependent variable was not analyzed by ANOVA. There was no effect of prenatal condition on the number of transitions (Figure 4C), but a significant decrease in number of transitions occurred when the object was present for both of the prenatal treatment conditions [$F(1,12)=17.3$, $p<0.01$]. This suggested that there was no significant difference between the prenatal treatment groups in general locomotor activity under these conditions. Time spent in the center with the object present was significantly greater for the FAE mice (Figure 4A), as indicated by the significant interaction between prenatal condition and object presence [$F(1,12)= 169.58$ $p<0.0001$], suggesting greater exploratory activity.

Effect of saccharin fading ethanol exposure paradigm on delay fear conditioning.

FAE mice and control pups were reared to adulthood and trained using a delay conditioning paradigm. Twenty-four hours later, mice were assessed for their conditioned responses to

the context and to the CS by measuring freezing behavior in response to reintroduction into the context in which conditioning occurred and exposure to the CS in a novel (altered) context (Figure 5). As can be seen in Figure 5, FAE mice froze less to the context than did saccharin controls, while freezing to the auditory CS was nearly identical in the two groups. An ANOVA revealed a significant effect of prenatal treatment condition [$F(1,36)=18.3$, $p<0.001$] and a significant interaction between prenatal treatment and stimulus condition (tone versus context) [$F(1,36)=19.5$, $p<0.001$], indicating the FAE mice froze less to the context than saccharin mice but there were no differences between the prenatal treatment conditions in freezing to the tone.

DISCUSSION

Our goal was to develop a prenatal alcohol exposure model that would: 1) produce a voluntary and stable drinking of moderate concentrations of ethanol throughout pregnancy, 2) not significantly affect maternal care, and 3) replicate the behavioral effects found previously in the rat moderate alcohol drinking model. We believe that we have developed a mouse model of FAE that accomplishes these goals. Using a moderate dose of ethanol (10%, w/v), this paradigm did not affect fetal birth weight, litter size, maternal care, or locomotor activity of the offspring, yet, characterization of the behavioral phenotype of the offspring revealed deficits in contextual fear conditioning, as well as increased novelty exploratory behavior. In addition, we found no effect on pup mortality rates. This is in sharp contrast to earlier mouse models which reported a marked decrease in the number of viable pups among the alcohol diet groups (Boggan et al., 1979; Randall and Taylor, 1979;Fish et al1981).

The paradigm described herein developed drinking behavior in females prior to impregnation. Thus, stable drinking patterns and blood levels were established before the beginning of pregnancy. We believe that this more closely models human alcohol drinking patterns than do paradigms in which alcohol consumption is initiated once pregnancy has been established.

An additional goal of our model was to permit the mothers to care for their own pups, thereby reducing the stress of cross-fostering. In order to accomplish this, we needed to reduce the alcohol concentration after the pups were delivered as quickly as possible, but without precipitating any withdrawal signs. In several pilot studies we determined that reducing both the ethanol and saccharin concentrations by one half every two days was slow enough to prevent any signs of handling-induced seizures or maternal neglect. While gross measurements, such as pup retrieval times, pup weights, pup grooming and litter sizes, suggest that maternal care was appropriate; other subtle differences between 10% ethanol and

saccharin drinking mothers are likely to exist. In humans, it is well known that maternal alcohol consumption may slightly reduce milk production and that some of the alcohol consumed is transferred in the milk (for review, Mennella, 2001). Using a rat model of prenatal and postnatal 20% alcohol exposure, Murillo-Fuentes et al. (2001), found no significant effect of ethanol on birth weight, but milk consumption and suckling behavior were reduced in pups whose mother continued to consume 20% ethanol. In our model, the nursing mothers were tapered off the ethanol solution and this may be why we did not see a significant difference in the pup weight between the 10% ethanol and saccharin drinking control groups.

Performance in the novel object task is an indication of exploratory responsiveness to novel stimuli, and, while it is not a standard measure of anxiety, results of this task are influenced by anxiety. The test begins similar to a standard open field, spontaneous locomotion study, where entries into the center of the field are suggestive of lower levels of anxiety in the mouse. Mice generally display neophobia and, though attracted by novel objects, typically keep a safe distance from them (see File, 2001 for review). Mice that perform with reduced anxiety on other tests (e.g., elevated plus and social interaction) typically will spend more time in close proximity of the novel object. An increase in number of entries into close proximity to a novel object indicates increased inquisitive behavior, while increased time spent near the novel object indicates inspective behavior (Grailhe et al. 1999; Dellu et al 2000). In our study, the 10% ethanol mice reacted to the presentation of a novel object with increased inspective behavior compared to saccharin animals. It is not likely that the increase in time spent in proximity to the novel object was due to decreased locomotor activity, since entries into the center and the number of transitions in the open field were not different in the 10% ethanol and saccharin mice. The higher level of inspective behavior may be due to a failure in the formation of associations and information processing, as suggested by their impaired performance in the

fear conditioning task (see below). Another interpretation of these data is that the 10% ethanol mice display a reduced level of anxiety.

The hippocampal formation has long been a focus of studies attempting to uncover the basis of ethanol-induced learning and memory deficits. In rodents, FAE is associated with impaired learning and/or memory in a variety of hippocampal-dependent tasks, including: radial arm maze paradigms (Reyes et al. 1989), the Morris water maze task (Gabriel et al. 2002, Savage et al. 2002), and avoidance learning (Furuya et al. 1996). In a recent group of studies, we found that rats that were exposed prenatally to moderate levels of ethanol display deficits in contextual fear conditioning, but not fear of a discrete CS (Weeber et al. 2001). Similar to the findings of Weeber et al., (2001) using the rat model, the 10% ethanol mice displayed significant deficits in single trial fear conditioning (Figure 5). However, with the mouse model, a more robust attenuation of fear conditioned freezing was seen here (Figure 5) compared to that reported in rat (Weeber et al., 2001). Impaired contextual conditioning, but unaltered CS (tone) conditioning, indicates that FAE disrupts hippocampal functioning, while leaving amygdalar functioning intact. The hippocampal formation plays a critical role in the consolidation and expression of contextual fear memory, whereas the amygdala plays an essential role in the acquisition and consolidation of information about both the elemental CS and the training context, as well as the expression of both CS fear and contextual fear responses (see Weeber et al 2001 for discussion). These results indicate that the 10% ethanol mice have impaired formation of the association between the context and the US. Identification of a learning/memory deficit in adult offspring demonstrates that the mouse, as well as the previously described rat, FAE model produces learning and memory impairments which persist into adulthood, similar to the clinical course of FASD in humans. It is interesting to note that Green et al. (2002) reported that eyeblink delay conditioning is

impaired in rats that were exposed to alcohol as neonates, approximating the human third trimester period. Thus, FAE-induced deficits in associative learning measured using delay conditioning paradigms are reproducible across species and appear to be a useful behavioral endpoint for the study of the effects of therapeutic interventions on FAE-induced learning and memory deficits.

An advantage of this mouse paradigm is that it will support drinking of high, moderate and low ethanol amounts in a voluntary design. The procedure that we describe could be modified to study effects of "binge"-like drinking, as we have found that, by limiting alcohol availability to a two-hour period, females will display higher alcohol drinking. For example, we have found that mothers will drink saccharin sweetened 15% (w/v) ethanol in a two hour restricted access paradigm and generate blood alcohol levels of 150-180 mg/dL. In addition, the use of a voluntary drinking approach can be employed for the study of the effects of high blood alcohol levels as it avoids the problems of malnutrition that are a consequence of using higher concentrations of ethanol in liquid diets or the stress associated with oral intubation, which is often employed to administer larger quantities of ethanol to dams.

The mouse FAE model described herein should prove useful in studies that exploit the availability of genetically modified, as well as several well characterized, mouse strains to assess the interplay between different genes in determining susceptibility to and the outcome of FAE. The studies presented here were performed using B6SJL/F1 mice because of our interest in developing a model that could be used with genetically altered mouse populations. However, it was important to demonstrate that similar results could be obtained in a genetically stable inbred strain. In a series of pilot studies we have obtained similar results on drinking levels, litter sizes and pup weights using the C57 BL/6 J mice (data not shown). It is important

to note that, though the mouse does offer certain important advantages over other animal models of FAE, it will be important to study outcomes in a variety of models.

Finally, it is possible that one model may more closely replicate certain characteristics of human FASD (e.g., attention deficits), while another model more closely reproduces other characteristics (e.g., impaired spatial learning). Extensive characterization of the effects of FAE on various neurochemical, electrophysiologic and behavioral endpoints in other models has identified several targets for therapeutic intervention, which could be tested in our mouse model. Further, thorough characterization of the effects of FAE in different animal models may identify distinct endpoints, which, in light of known differences in rodent brain neurochemistry and neurophysiology (e.g., differences in hippocampal GAP-43 expression, McNamara et al,1996), may provide clues to the effects of genetic influence on the effects of FAE, and, thus identify therapeutic targets.

In conclusion, we describe the development and initial characterization of a mouse model of FAE. This model complements and extends other available models for the study of FAE. The paradigm that we employed will facilitate the study of the impact of FAE without negatively influencing diet, natural consumption routes, or requiring surrogate maternal care, all of which are thought to contribute to maternal stress. The mouse FAE model allows the use of genetically altered mice, as well as genetically defined inbred mouse strains, for identifying risk factors that are associated with FASD cognitive deficits.

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TABLE 1

Maternal weight change, food consumption, pup weights, litter size, from 4 separate studies (Values are mean ± SEM, n= 6 mothers per study)

Treatment Condition	Change in maternal weight (g)	Daily food consumption (gm)	Pup weight (g)	Litter size
10% (w/v) EtOH in saccharin	+1.08 (± 0.2)	4.4 (±0.38)	4.3 (±0.4)	8.4 (±1.3)
Saccharin only	+1.02 (± 0.4)	4.2 (±0.13)	4.1 (±0.2)	8.2 (±2.0)

TABLE 2

Pup retrieval time, percent time nesting and pup grooming from 4 separate studies (Values are mean ± SEM, n= 6 mothers per study)

Treatment Condition	Pup retrieval time (sec)	Percent time on nest (min/2hour observation)	Percent time grooming pup
10% (w/v) EtOH in saccharin	62 (±8)	52 (±3)	7.2 (±1.0)
Saccharin only	57 (± 1)	49 (±4)	9.7 (±0.9)

TABLE 3

Average adrenal weights and plasma corticosterone levels in offspring from saccharin and ethanol drinking dams taken at 0900-1000 hr. Values are mean (± SEM, n=10). Asterisk indicates statistically different from saccharin females, $p<0.03$.

	Average single Adrenal Weight (mg)	Total Adrenal wt/ body wt x 100	Plasma Corticosterone level ug/dl
Saccharin female	3.10 (± 0.25)	0.0261 (± 0.003)	3.00 (± 0.4)
10% EtOH female	2.74 (± 0.20)	0.0253 (± 0.004)	5.06 (± 0.4) *
Saccharin male	1.46 (± 0.18)	0.0196 (± 0.009)	2.78 (± 0.3)
10% EtOH male	1.35 (± 0.21)	0.0163 (± 0.006)	3.30 (± 0.4)

FIGURE LEGENDS

FIGURE 1

The effect of the saccharin fading ethanol exposure paradigm on food and fluid consumption. In panel A, the amount (mean ± SEM, n=12) of food consumed by the female mice assigned to the ethanol drinking condition is presented. Consumption of the standard breeder block chow was measured every day for a total of 10 days while saccharin and ethanol were introduced. Panel B presents the volumes (mean ± SEM, n=12) of fluid consumed from the water (square symbols) and saccharin or saccharin/ethanol tubes (triangle symbols) for a total of 10 days for the same set of female mice.

FIGURE 2

The effect of the saccharin fading ethanol exposure paradigm on fluid consumption during pregnancy.

Figure 2 presents the two day total amount (mean ± SEM, n=12) of water only (squares) and total fluid (triangles) consumed by preganant dams in the saccharin drinking group (filled symbols) and the 10% w/v ethanol drinking (open symbols) conditions. Water and total fluid consumption (two day totals; mean ± SEM, n=12) are given from day 2 – day 14 of their pregnancies.

FIGURE 3

Effect of saccharin fading ethanol exposure paradigm on blood alcohol levels

Blood alcohol level determinations from saphenous vein blood of dams drinking 10% (w/v) ethanol in 0.06% saccharin across a 24 hour access period. Each point is mean ± SEM from

6-8 dams per time point. Each dam is represented at no more than 3 time points. Solid horizontal bar indicates the lights on period.

FIGURE 4

Effect of saccharin fading ethanol exposure paradigm on novelty exploration.

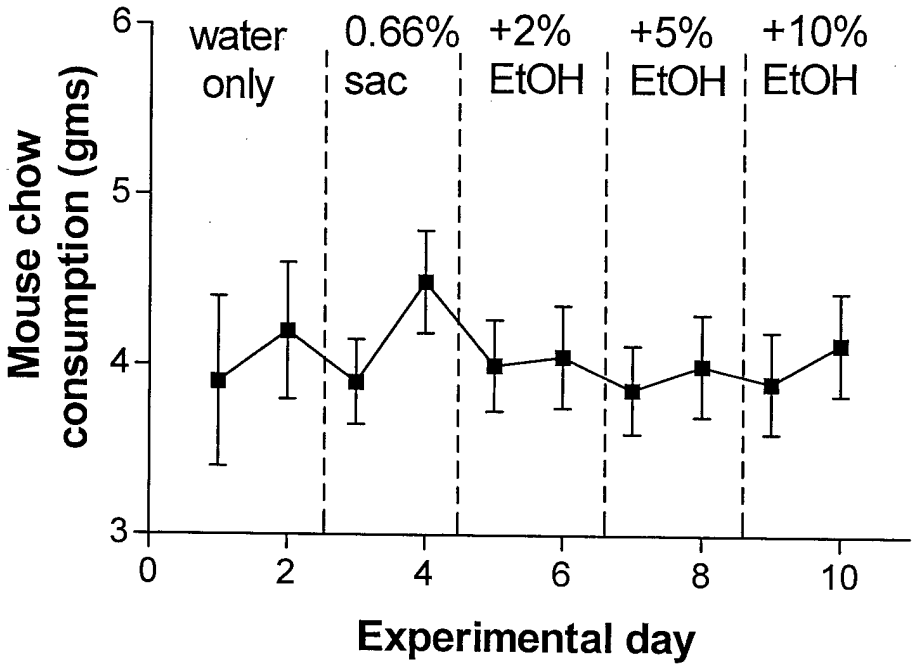
Novelty exploration was measured in adult male mice and expressed as mean (± SEM, n= 7 per group) time spent in the center (within 5 cm of the object, panel A), number of entries into the center area (panel B) and number of transitions across the quadrants of the open field (panel C). Data are presented as activity during the first 5 minutes without the object present in the first pair of bars and the behavioral activity during the last 5 minutes while the novel object was present in the last pair of bars. Asterisk represents $p<0.01$ by ANOVA.

FIGURE 5

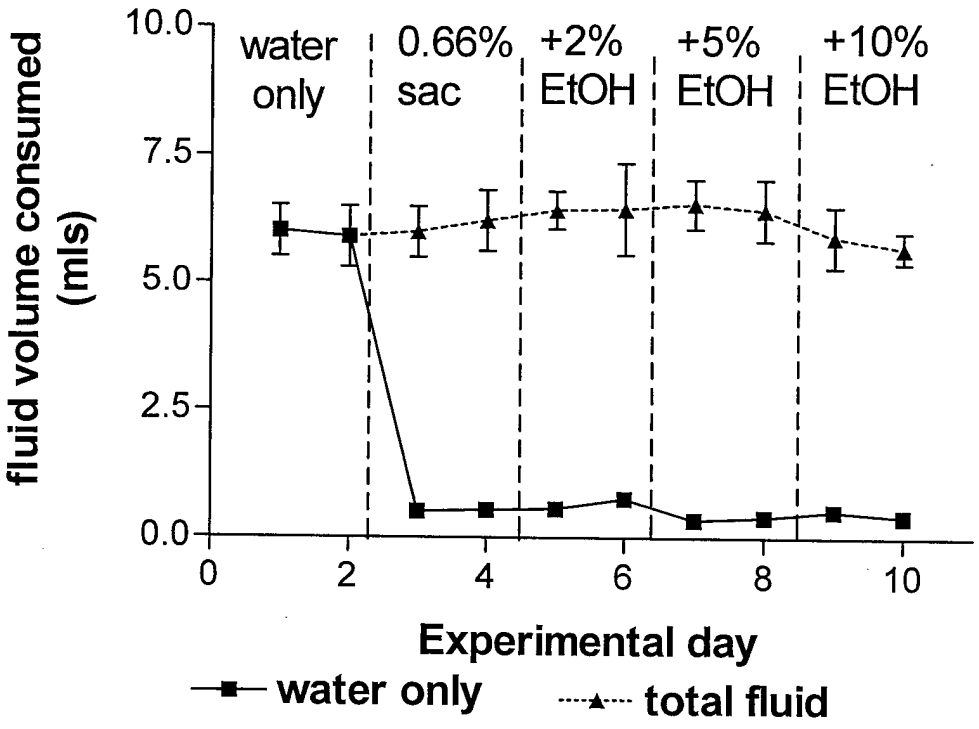
Effect of saccharin fading ethanol exposure paradigm on contextual fear learning and memory.

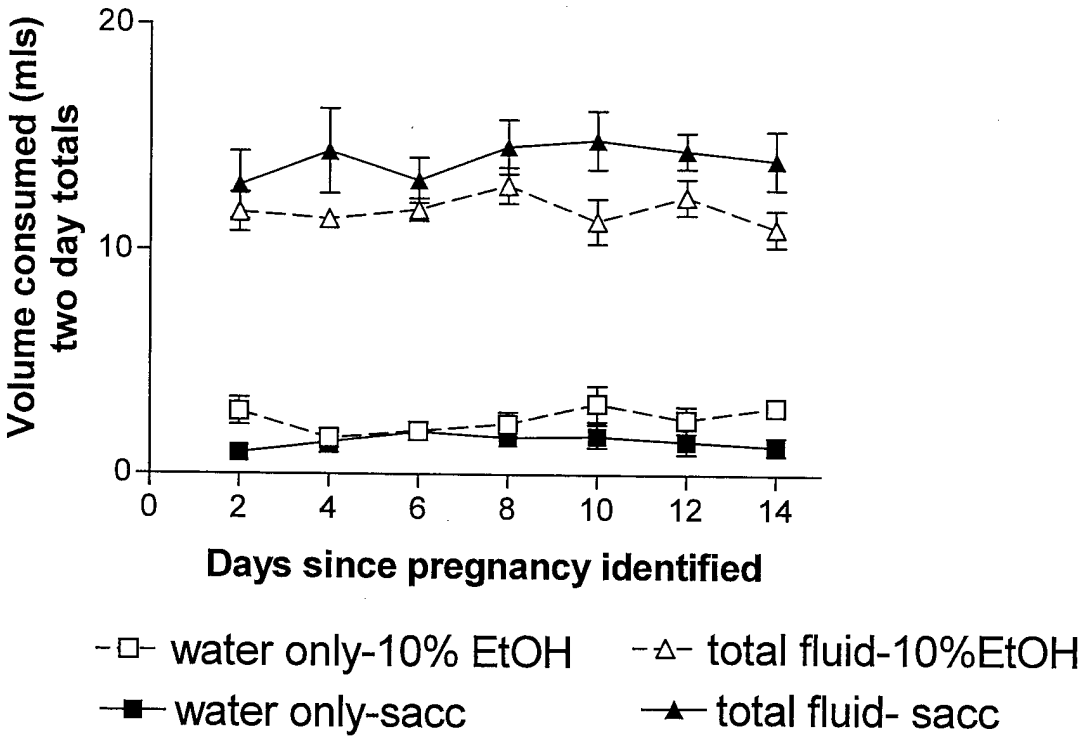
Freezing to the training context (top) and to the conditioned stimulus in a novel context (bottom) in saccharin control and 10% EtOH mice trained with a paired tone-shock. Adult offspring of dams consuming either 0.066% saccharin (Control; n=10) or 10% (w/v) ethanol in 0.066% saccharin (10% EtOH; n=10) were trained using a delay fear conditioning paradigm. Panel A : Twenty-four hours after training, the animals were returned to the training context and the conditioned response (freezing) was assessed. Data are expressed as the % freezing (# of freezing intervals ÷ total intervals). Panel B: Freezing to the CS tone was measured in a novel context. Data are expressed as described above. Asterisk represents $p<0.001$ by ANOVA.

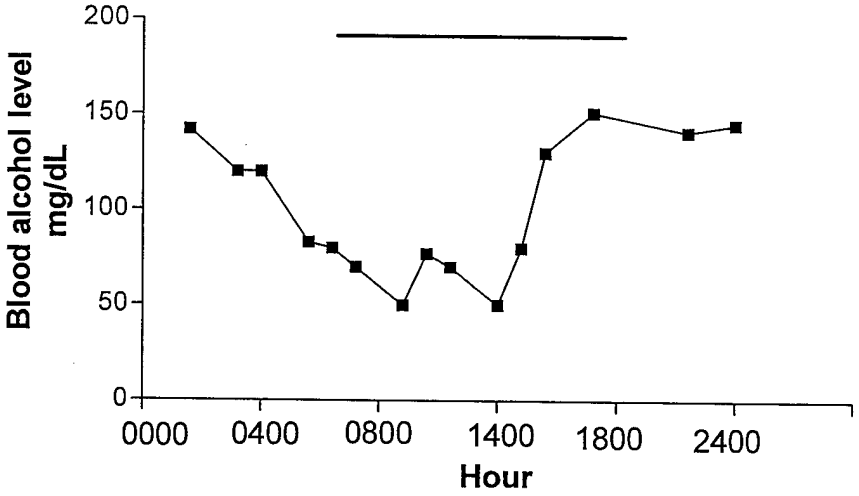
A.


water only
0.66% sac
+2% EtOH
+5% EtOH
+10% EtOH
Mouse chow consumption (gms)
6
5
4
3
0
2
4
6
8
10
Experimental day

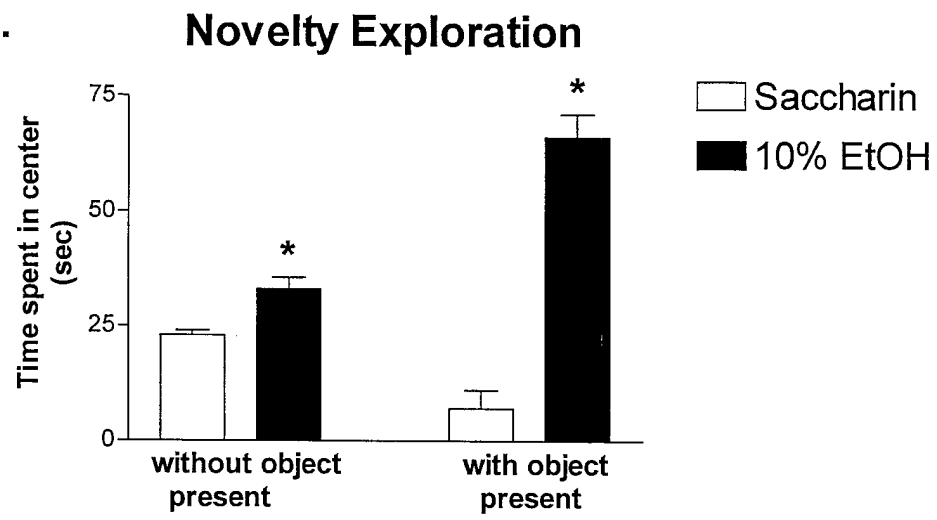
B.


water only
0.66% sac
+2% EtOH
+5% EtOH
+10% EtOH
fluid volume consumed (mls)
10.0
7.5
5.0
2.5
0.0
0
2
4
6
8
10
Experimental day
water only
total fluid

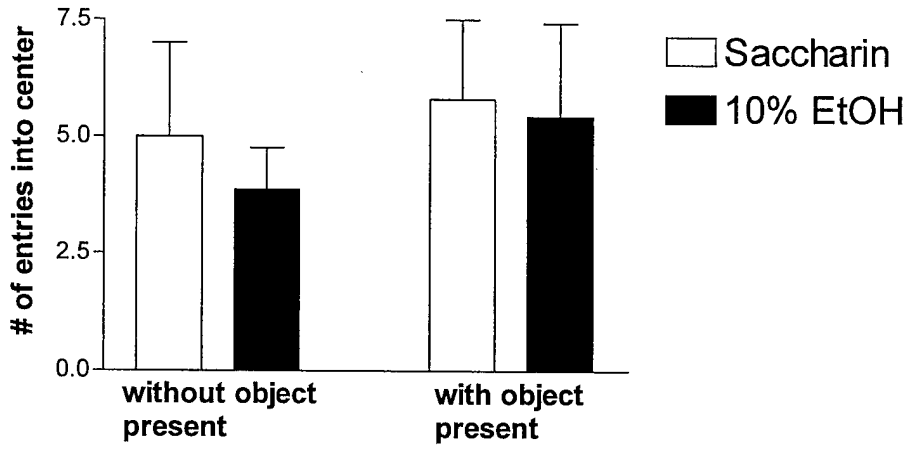

Volume consumed (mls)
two day totals
20
10
0
0
2
4
6
8
10
12
14
Days since pregnancy identified
water only-10% EtOH
total fluid-10%EtOH
water only-sacc
total fluid- sacc


200
150
100
50
0
Blood alcohol level
mg/dL
0000
0400
0800
1400
1800
2400
Hour

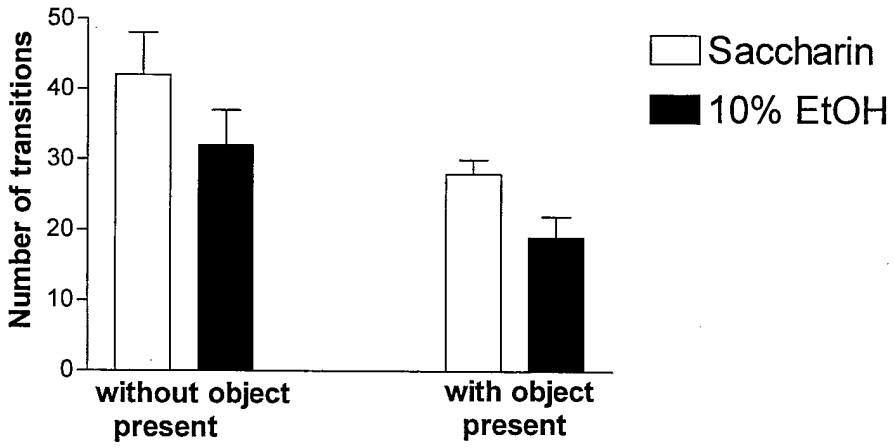
A.


Novelty Exploration
Time spent in center (sec)
75
50
25
0
*
*
without object present
with object present
Saccharin
10% EtOH

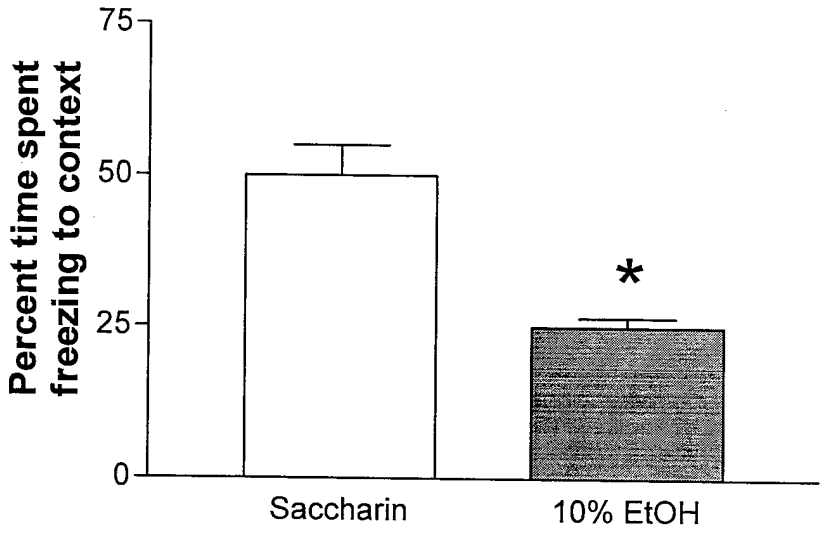
B.


# of entries into center
7.5
5.0
2.5
0.0
without object present
with object present
Saccharin
10% EtOH

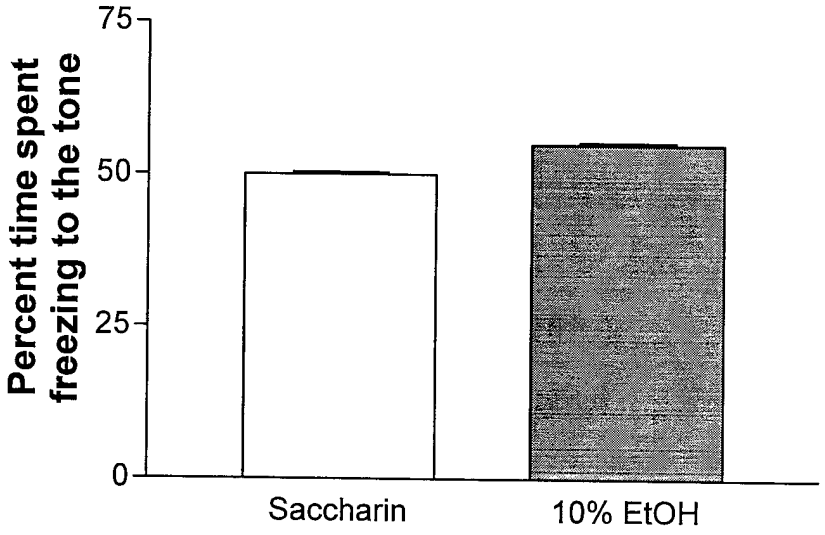
C.


Number of transitions
50
40
30
20
10
0
without object present
with object present
Saccharin
10% EtOH

A.


Percent time spent freezing to context
75
50
25
0
*
Saccharin
10% EtOH

B.


Percent time spent freezing to the tone
75
50
25
0
Saccharin
10% EtOH